CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

Vol. 11 Hormones in Blood

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VOLUME 11

Hormones in Blood

Editors for the Ciba Foundation

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O.B.E., M.A., M.B., B Ch.

and

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THE CIBA FOUNDATION

r the Promotion of International Co-operation in Medical and Chemical Research

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PREFACE

UNDER its eminent Trustees, the Ciba Foundation is engaged in a number of activities with the purpose of improving co-operation in medical and chemical research between workers in different countries and different disciplines. At its house in London the Foundation provides accommodation for scientists, organizes conferences, conducts a medical postgraduate exchange scheme between Great Britain and France, arranges a variety of short informal discussions, awards two annual lectureships, is building up a library service in special fields, and assists international congresses and scientific institutions and societies.

This volume contains the proceedings of the forty-third small international conference, each of which has lasted two to four days, held at the Foundation since 1950. It was the seventeenth conference on an endocrinological subject, proposed in this instance by Professor F. G. Young. Its organization was mainly in the hands, for the first time, of Dr. Genese, who received much helpful advice from Professor Young, Dr. A. S. Parkes, and Dr. J. A. Loraine.

Dr. Parkes very kindly acted as its Chairman, a rôle he had filled with distinction several times before at the Foundation, but never more effectively and delightfully than on this occasion.

The Group was, as usual at the Ciba Foundation, much restricted in size, partly because accommodation is limited, but chiefly because it is found that useful exchanges and arguments can best be obtained when the members can easily and quickly come to know each other well.

The senior Editor wishes to place on record here the debt of the Foundation and its guests to Miss Elaine Millar, who now leaves to take up work in the United States. This volume is the seventh she has edited for the Foundation in the past

two and a half years. Both Editors hope it will prove acceptable and informative to the original participants in the colloquium and to many readers throughout the world who could not be present in person.

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List of those participating in or attending the Colloquium

"Hormones in Blood"

18th–22nd February, 1957

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R. BORTH	Clinique Universitaire de Gynécologie et d'Obstétrique, Geneva
P. M. BOTTARI	Faculté de Médecine et de Pharmacie, University of Brussels
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A. E. KELLIE	Courtauld Institute of Biochemistry, Middlesex Hospital, London
J. A. LORAINE	Clinical Endocrinology Research Unit, University of Edinburgh
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GABRIELLA MORREALE DE ESCOBAR	Medical Research Centre, Spanish National Research Council, Granada, and Dept. of Internal Medicine, State University of Leiden

CHAIRMAN'S OPENING REMARKS

A. S. Parkes

There are two reasons why I have a special interest in this colloquium. In the first place, I have a tenuous connection with the word "hormone", arising from the fact that as a young post-graduate student I was interviewed for my first job by E. H. Starling, who was then Professor of Physiology at University College. I was well aware of Starling's pre-eminence as a physiologist; I was not aware at the time that about ten years before he had brought into use the word "hormone". The somewhat curious history and the inadequacies of the word as applied to the internal secretion of the endocrine organs were discussed fully by Sharpey-Schafer in 1924 and need not concern us now. The relevant point is that Starling defined a hormone as a substance produced in one organ of the body and carried by the circulating blood to another organ in which it evokes a response. I think it is important that we should remember this original definition. In the case of the secretion of the gonads, with which much of my own work has been concerned and which will no doubt feature to some extent in this colloquium, there has been a tendency to forget it. A hormone is not something that occurs in the urine or something knocked up by a chemist in his laboratory; a hormone is something that goes round in the blood to act in another part of the body. For this reason I like the title of this colloquium.

Unfortunately, the detection of the gonadal hormones in the circulating blood was very difficult in the early days, and attention was readily diverted from this awkward problem by the discovery that large amounts of analogously active substances occurred in the urine. This concentration of work on excretion products enabled the chemist to make his vital

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THE STATE AND CONCENTRATION OF THE NEUROHYPOPHYSIAL HORMONES IN THE BLOOD

H. HELLER

Department of Pharmacology, University of Bristol

IT is known that certain steroid hormones (Szego and Roberts, 1946, 1947; Daughaday, 1956) and the thyroid hormones are bound or partially bound by plasma proteins. The peptide hormones have been little investigated but protein binding of neurohypophysial principles has long been suspected. The problem is complicated by the possibility that the hormones reach the blood not as the free octapeptides but in a storage form linked to a protein of hypothalamic or glandular origin (van Dyke, Chow, Greep and Rothen, 1942; Acher and Fromageot, 1957). However, so far as "exogenous" vasopressin is concerned, Ginsburg and Heller (1953*a*) found in animals in which the main sites of clearance, namely the kidneys and the splanchnic vascular bed, had been eliminated, that intravenously injected antidiuretic hormone (ADH) left the blood more slowly than would be expected from a freely diffusible compound of the molecular weight of about 1,000. Binding of vasopressin and oxytocin is also suggested by numerous reports (de Wesselow and Griffith, 1934; Broun and Scheiner, 1935; Levitt, 1936; Heller, 1937; Croxatto, Andrade and Barnafi, 1952) that the ultrafiltrability of the neurohypophysial principles is decreased when they are mixed with plasma or blood. Heller and Lederis (unpublished) have recently done some experiments in which they compared the rate of dialysis of the pressor and oxytocic principles from 0·9 per cent sodium chloride solution with that from human plasma. They used Pituitrin (Parke, Davis & Co.) and synthetic oxytocin and found in all instances that the activity

contribution to the subject and to supply the biologist with large amounts of active substances, but it confused sadly the biological picture. A few years ago in referring to this matter at the Royal Society of Medicine I recalled that Dobriner's methods of examining urinary steroids resulted in the production of up to 3000 fractions from a single specimen of urine and urged a policy of "Back to Blood". This is the second reason for my interest in this colloquium and no doubt the main reason for the invitation, which I much appreciate, to take the chair.

This is the forty-third Ciba Foundation international conference, and I have been involved in one way or another with many of them. It is difficult to say what exactly is the keynote of these most pleasant and useful meetings, but I think it can be said that, whereas at these conferences we take our science seriously, we do not take ourselves too seriously. In this way the Ciba Foundation conferences have acquired a most happy reputation, which I am sure will be more than maintained on the present occasion.

deliberately overhydrated. Normally, therefore, one would expect to find some ADH in the blood but one must also expect fluctuation in the hormone concentration (*a*) due to the

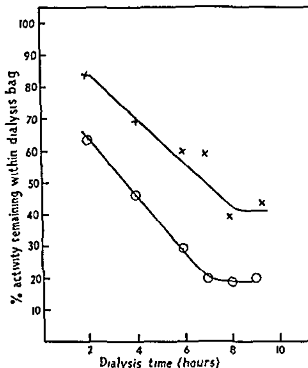


FIG. 1 Dialysis to equilibrium of synthetic oxytocin (Syntocinon, Sandoz). x——x in human plasma, O——O in Krebs-Eggleston phosphate-saline buffer. Initial concentration of oxytocin in dialysis bag : 100 mu. = 0·2

published)

diurnal rhythm already mentioned, (*b*) in relation to fluid ingestion and (*c*) as a result of seasonal influences. In hot weather there is likely to be an increased release of ADH (Heller and Smirk, 1932; Bonvallet, Dell and Stutinsky, 1948; Itoh, 1954; Macfarlane and Robinson, 1957). What then is

disappeared more slowly from plasma than from saline. Inulin, on the other hand, i.e. a substance which is known (Shannon and Smith, 1935) not to be bound by plasma proteins, could, under the same experimental conditions, be shown to dialyse from saline and plasma at about the same rate. In the experiments so far mentioned the dialysis was done against running water. Similar results were obtained when the hormones were dialysed to equilibrium against a fixed volume of Krebs-Eggleston (1940) solution (Fig. 1). Extensions of these experiments are planned.

Turning now to the discussion of the concentration of the neurohypophysial principles in the blood, the first question one naturally asks is: What is the normal concentration of the posterior pituitary hormones in the blood? There are several reasons why it is difficult to answer this question. First, "normal" has to be defined in terms of the physiological function of the hypothalamo-neurohypophysial system. This is relatively easy for the antidiuretic hormone. We know enough to assume that in the healthy adult mammal, the osmolarity of the urine during the 24 hours of the day will rarely reach values comparable to those in a patient with diabetes insipidus with free access to water (Heller, 1944). It has been shown (Sirota, Baldwin and Villarreal, 1950; Mills, 1951; Lewis, Lobban and Shaw, 1956) that the diurnal changes in urine flow are almost wholly attributable to variations in the tubular reabsorption of water and that the 24-hour cycle is maintained even when the subject's sleeping habits are changed and his fluid intake at night equals that of the day. This suggests strongly that the urine volume is controlled by a continuous release of ADH but that the amounts of hormone secreted are influenced by an inherent hypothalamic rhythm. Superimposed on the overall diurnal changes is a more transient physiological situation, namely the intake of fluid to satisfy thirst. This spontaneous water intake can only rarely be large enough to inhibit the secretion of ADH by the healthy mammal completely. It cannot be compared with the familiar antidiuretic assay procedure when the animal is

animal by the subcutaneous or intraperitoneal injection and hence liberation of endogenous ADH may be one factor. The presence of potentiating substances which delay absorption (Noble and Taylor, 1953) may be another, and the occurrence of substances like 5-hydroxytryptamine, which seems to be more potent on intraperitoneal than on intravenous injection (Erspamer and Sala, 1954), may be a third.

Other reasons why incomprehensibly large amounts of antidiuretic activity have sometimes been found in blood—even when an intravenous assay method was used—are connected with the experimental conditions under which the blood samples were obtained. Ether (Ginsburg and Heller, 1952, 1953*a*; Ames and van Dyke, 1952; Eversole and Giere, 1954) and pentobarbitone (de Bodo and Prescott, 1945; Ginsburg and Brown, 1956), for example, have been shown to stimulate the neurohypophysis; so does the removal of relatively small volumes of blood by cardiac puncture (Ginsburg and Heller, 1952, 1953*a*; Ames and van Dyke, 1952) and so, at least in the rat, does massive haemorrhage (Ginsburg and Heller, 1953*a*; Ginsburg and Brown, 1956). Moreover, as already stressed, pain or discomfort of the patient or animal at blood removal has to be strictly avoided. For instance, it seems to me quite possible that pain or excitement may be the explanation why we occasionally found determinable amounts of antidiuretic activity when we obtained blood by internal jugular puncture even though the skin of our subjects had been infiltrated with procaine and they appeared to be outwardly calm.

Summarizing then the "acceptable" data, it would appear that in the temperate zone in man and in the rat the normal concentrations of ADH in peripheral blood are so low that they cannot be estimated with the techniques at present at our disposal. This conclusion—at least for the rat—is consistent with a calculation based upon an estimate of the rate of liberation of ADH in normal animals and its rate of clearance from the circulation. According to Dicker (1954) 8–15 μu./min./100 g are secreted. It can be calculated from this and from the constant obtained by Ginsburg and Heller (1953*b*)

the order of magnitude of these "normal" levels? A survey of recently published results of estimations of antidiuretic activity in human and animal blood shows that they can be roughly divided into two classes. There are firstly some authors who have found relatively large amounts of antidiuretic activity in the blood. Mirsky, Stein and Paulisch (1954) for instance found activity equivalent to 184 ± 14 µu. (microunits) vasopressin per ml. normal rat plasma, and Lloyd and Pierog (1955) reported values of up to 400 µu. For human blood, antidiuretic values equivalent to several 100 µu./ml. have frequently been reported and Perry and Fyles as recently as in 1953 published values which, if due to the posterior pituitary ADH, would be equal to about 10,000 µu./ml. serum.

In contrast to these findings are the reports of Ames and van Dyke (1952) who found less than 20 to 40 µu./ml. serum from blood obtained by decapitation of rats in normal water balance, and of Ginsburg and Heller (1953*a*) who found less than 100 µu./ml. plasma from blood which had been collected from the external jugular vein of normal rats. The latter finding gains in significance considering that in the rat the external jugular vein is the main drainage of the pituitary. In human beings in which the venous outflow from the gland is mainly carried by the *internal* jugular vein, antidiuretic activity was either so low that it could not be estimated, i.e. it was lower than 12–20 µu./ml. jugular plasma (Heller and Schnieden, 1955; Bisset, Lee and Bromwich, 1956) or in a few instances in the region of 10–20 µu./ml.

Several reasons can be advanced for this striking discrepancy in results. First of all, it has by now been shown repeatedly and unequivocally that blood, plasma or serum injected subcutaneously or intraperitoneally into rats (which are the most commonly used animals for this kind of assay) may, when standardized against Pitressin, give much higher results than intravenous injections of the same material into rats, dogs or human beings. Up to 25-fold differences have been recorded (Ames and van Dyke, 1952; Lewis, 1953). Why they arise is not quite clear. Pain or irritation of the conscious

into rats. Certain results of Ames and van Dyke (1952) in the Kangaroo rat should, I think, also be mentioned in this context. This species of desert animal has been shown never to drink water spontaneously and the hormone content of its neurohypophysis is exceptionally high. It can concentrate its urine to values well above those obtained in the laboratory rat (Schmidt-Nielsen *et al.*, 1948) and excretes normally large amounts of antidiuretic activity in the urine (Ames and van Dyke, 1950). It may therefore be said to live in a state of physiological dehydration. Plasma antidiuretic values in this species in series of 20 tranquil animals were: 50 to 200 μu./ml. in 12 and less than about 50 μu./ml. in 8.

There is no doubt that the "unphysiological" stimuli already mentioned can raise the antidiuretic activity of the blood to prodigious levels, for example up to 26,000 μu./ml. external jugular blood in the rat (Ginsburg and Brown, 1956). It is as yet difficult to say whether we are here dealing with an "emergency function" of the posterior pituitary either because the large amounts of active principles released are aimed at certain peripheral target organs or whether we are concerned with an "overspill" from the hypophysial portal circulation into which these hormones, together perhaps with other hypothalamic principles, have been released to stimulate the anterior lobe and thus indirectly the adrenal cortex, or whether both possibilities apply. As pointed out by Ginsburg (1957), the amounts of vasopressin released by haemorrhage in the rat are certainly high enough to suggest that the hormone could have a sustaining effect on blood pressure. If these observations can be extended to other animals and man, they would, I suppose, furnish us with an explanation why, compared with the minimum antidiuretic dose, such very large quantities of hormone are held in readiness in the neural lobe.

Methods to estimate the oxytocic hormone in the blood are not as yet entirely satisfactory. However, I should like to mention some results of Fitzpatrick and Hughes (1957) because the animals from which the blood was obtained were as nearly normal as such experiments permit. They collected

in the equation for the clearance of ADH that the corresponding concentration of the hormone in the blood would be 0·8 *to* 3·2 μu./ml. or 5 to 19 μu. *in the total blood volume.* This latter figure seems reasonable since it is of the same order as the minimum antidiuretic dose.

Lack of information on the rate of clearance of the antidiuretic hormone prevents similar calculations for species other than the rat but there are some indications that dehydration may raise the hormone concentration in the blood to levels which can be determined. *Blackmore and Chester (1956) withdrew water from dogs for 24 hours and on intravenous* injection of *plasma* samples into rats *anaesthetized* with ethanol, reported antidiuretic activity equivalent to 45–105 μu. vasopressin per ml. plasma. Buchborn (1956) quite recently has published an impressive curve in which he plots plasma antidiuretic levels against serum osmolar concentration. As predictable from Verney's (1947) classical work, a significant correlation was obtained. *It is of great clinical interest since it may explain why such varying values for blood antidiuretic* activity have been obtained in certain oedematous conditions. Heller and Schnieden, for instance, in a small series of patients with liver cirrhosis and ascites (Heller and Schnieden, unpublished) and in some infants with Kwashiorkor (Heller, 1956) found, in blood samples obtained by internal jugular puncture, concentrations which, in different patients, varied *from less than 11 to 152 μu./ml. plasma. The influence of* excitement again cannot be discounted, but it may be that these figures reflect differences in plasma osmotic pressure or distortions of the extracellular fluid phase. However, Buchborn's values at normal serum molarity (300–310 mOsm./l.) seem somewhat high considering that Lewis (1953), using an impeccable method *of assay, found the activity of human plasma, after* 12 hours dehydration, to be well below 100 μu./ml. Buchborn used a new method of assay (he injected his test material into the lymph sac of toads) and it cannot as yet be excluded that his antidiuretic effects were somewhat "potentiated"; in a similar manner as after intraperitoneal *injection*

uterine activity (see Fitzpatrick, 1957) during the oestrous cycle would be compatible with periodic changes in the release of the two neurohypophysial principles.

So much for the rôle of oxytocin in the female. But we are

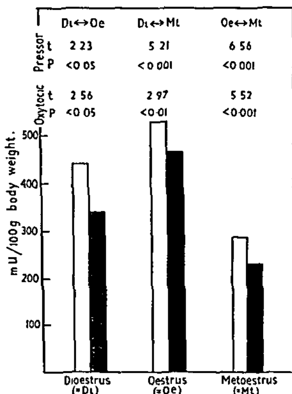


FIG. 2 Pressor (▭) and oxytocic (▬) activity in the pituitary glands of adult female rats at different stages of the oestrous cycle (Heller and Lederis, unpublished).

still faced with the puzzling fact that the pituitary of the male contains large quantities of oxytocin. Is there any indication that oxytocin is needed by the male? There may be one which had recently come to light; Brooks and Pickford (1957) have reported that the intravenous injection of oxytocin into dogs

blood from an indwelling catheter in an external jugular vein of cows, extracted the blood by a modification of the method of Bisset and Walker (1954) and assayed the extracts on the superfused rat uterus. Oxytocic values ranging from 120–300 μu./ml. were obtained. The interpretation of the results of Bisset, Lee and Bromwich (1956) on blood samples collected from an internal jugular vein of patients at operation is—as the authors stress themselves—obscured by the fact that the donors were under the influence of a variety of drugs. But it is noteworthy that much more oxytocic than antidiuretic activity was found, a discrepancy which has also been noted whenever the release of neurohypophysial hormones has been investigated indirectly by their effect on the target organs (Harris, 1948; Cross, 1951; Abrahams and Pickford, 1954).

Another difficulty in discussing "normal" blood levels of the oxytocic hormone arises from the fact that the physiological premises for its release are less clear than those for the secretion of the antidiuretic-vasopressor principle. There is good evidence (see Cowie and Folley, 1957) for its release during lactation but some results have recently come to hand which suggest that there may also be variations in the release of oxytocin during the oestrous cycle and that therefore cyclical changes in the blood hormone level may have to be expected in the female. When estimating oxytocic and pressor activity in the pituitary of adult female rats, Heller and Lederis (unpublished) found that the glands of animals in metoestrus contained significantly less hormone than the pituitaries of rats in dioestrus or in oestrus. This applied to both the pressor principle and to the oxytocic principle (Fig. 2). Both hormones in the metoestrous glands were apparently decreased in much the same proportion, that is to say the ratio of pressor to oxytocic activity remained roughly the same. Smaller differences of yet uncertain significance were also found between dioestrous and oestrous glands. This work is still in a preliminary stage and results in other species are obviously needed, but the well-known changes in water metabolism (Krohn and Zuckerman, 1937; Pickford, 1956) and

BROUN, D., and SCHEINER, H. (1935). *C. R. Soc. Biol., Paris*, **119**, 1379.
BUCHBORN, E. (1956). *Klin. Wschr.*, **34**, 953.
COWIE, A. T., and FOLLEY, S. J. (1957). *In* The Neurohypophysis. London: Butterworth.
CROSS, B. A. (1951). *J. Physiol.*, **114**, 447.
CROXATTO, H., ANDRADE, F., and BARNAFI, L. (1952). *Acta physiol., Latinoamer.*, **2**, 142.
DAUGHADAY, W. (1956) *J. clin. Invest.*, **35**, 1428.
DICKER, S. E. (1954). *J. Physiol.*, **124**, 464
DICKER, S. E., and HELLER, H (1946). *J. Physiol.*, **104**, 353.
VAN DYKE, H. B., CHOW, B F., GREEP, R. O., and ROTHEN, A. (1942). *J. Pharmacol.*, **74**, 190.
ERSPAMER, V. J, and SALA, G. (1954). *Brit. J Pharmacol.*, **9**, 31.
EVERSOLE, W. J., and GIERE, F. A. (1954). *Fed. Proc.*, **13**, 42.
FITZPATRICK, R. J. (1957) *In* The Neurohypophysis. London: Butterworth.
FITZPATRICK, R. J., and HUGHES, J. M. (1957). *In* The Neurohypophysis. London. Butterworth.
GINSBURG, M (1957). *In* The Neurohypophysis. London: Butterworth.
GINSBURG, M., and BROWN, L M. (1956). *Brit. J. Pharmacol.*, **11**, 236.
GINSBURG, M., and HELLER, H. (1952). Cited by HELLER, H *In* The Suprarenal Cortex. London: Butterworth
[illegible]
HELLER, H. (1956). Cited by DAVIES, J N. P., *Amer. J. clin. Nutr.*, **4**, 539.
HELLER, H., and SCHNIEDEN, H. (1955) Cited by HELLER, H, *Mem. Soc. Endocrin.*, **5**, 25
HELLER, H., and SMIRK, F H (1932) *J. Physiol.*, **76**, 23.
HELLER, H., and ZAIDI, S. M. A (1957) *In* The Neurohypophysis. London: Butterworth.
ITOH, S. (1954). *Jap J. Physiol.*, **4**, 185.
KREBS, H. A., and EGGLESTON, L. V. (1940). *Biochem. J.*, **34**, 442.
KROHN, P L, and Zuckerman, S. (1937) *J. Physiol.*, **88**, 369
LEVITT, G (1936). *J. clin. Invest.*, **15**, 135.
LEWIS, A. A. G. (1953). *J. clin. Endocrin. Metab.*, **13**, 769.
LEWIS, P. R., LOBBAN, M. C, and SHAW, T. I. (1956) *J. Physiol.*, **133**, 659.
[illegible] **54**, 491.
NOBLE, R. L, and TAYLOR, N. B. G (1953) *J. Physiol.*, **122**, 220.
PERRY, W F., and FYLES, T. W. (1953) *J clin. Endocrin. Metab.*, **13**, 64

results in a marked increase of renal blood flow (diodone clearance) though glomerular filtration rate (GFR) remained unaffected. They showed further that under certain circumstances oxytocin increased renal sodium excretion. These findings resemble older results of Dicker and Heller (1946) in rats. After subcutaneous injections of Pitocin we found that, as in the dog, the diodone clearance was increased. We also found that Pitocin raised the urinary chloride excretion significantly. These results admit the tentative conclusion that oxytocin may be somehow concerned in the regulation of renal function and in particular in the control of electrolyte secretion.

I regret that so much of my discussion was devoted to the difficulties of estimating the level of the neurohypophysial hormones in the blood. But these difficulties do not prevent me from echoing our chairman's slogan "Back to Blood". This maxim is certainly very appropriate to the posterior pituitary hormones considering that we were recently able to show (Ginsburg and Heller, 1953*b*; Heller and Zaidi, 1957) that ADH, at least, is not only removed by but also partly metabolized in the kidney. Alterations in renal function and hence in the urinary excretion of the hormones may therefore mask changes in neurohypophysial function which would be revealed by estimating the hormone in the blood.

REFERENCES

ABRAHAMS, V. C., and PICKFORD, M. (1954). *J. Physiol.*, 126, 329.
ACHER, R., and FROMAGEOT, C. (1957). *In* The Neurohypophysis. London: Butterworth.
AMES, R. G., and VAN DYKE, H. B. (1950). *Proc. Soc. exp. Biol., N.Y.*, 75, 417.
ology, 50, 350.
Lancet, 2, 1129.
l., 126, 588.
ocrinology, 59, 493.
armacol., 85, 222.
(1948). *J. Physiol.*
Path. gén., 40, 120.
BROOKS, F. P., and PICKFORD, M. (1957). *In* The Neurohypophysis. London: Butterworth.

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REFERENCES

Abrahams, V. C., and Pickford, M. (1954). *J. Physiol.*, 126, 329.

Acher, R., and Fromageot, C. (1957). *In* The Neurohypophysis. London: Butterworth.

Ames, R. G., and van Dyke, H. B. (1950) *Proc. Soc. exp. Biol., N.Y.*, 75, 417.

[illegible]

Bonvallet, [illegible] *Path. gén.*, 40, 120.

Brooks, F. P., and Pickford, M. (1957). *In* The Neurohypophysis. London: Butterworth.

be demonstrated in extracts of kidney tissue a few minutes after the injection. This makes us believe that the kidney not only removes ADH from the blood, but also that some of the hormone is metabolized in that organ.

Querido: I asked the question because I think that part of the purpose

Finally it disappears from the kidney.

So we also have the impression that TSH is in some way picked up and metabolized in kidney tissue.

Heller: The situation is even more complicated than that in the case

been shown to inactivate vasopressin very effectively *in vitro*.

Ingbar: I wonder whether the differences in the assay that one gets depending upon the route of administration might be related to the variations in the state of hydration that are produced in the recipient animal.

Heller: This is certainly a point. The state of hydration has to be carefully watched. The sensitivity of the assay animals may be in-

Ingbar. I was thinking more of the differences in the state of hydration of the animal as a result of varying routes of administration, for example following intravenous and intraperitoneal injection of plasma. I do not know what volume of plasma you inject, but an intravenous injection of a considerable volume of plasma into the rat might produce considerable overhydration.

Heller: Yes. May I, in that connection, direct your attention to a text-figure which we have published in the Memoirs of the Society for Endocrinology, No. 5, in which we show that the antidiuretic response to a given dose of Pitressin varies according to the volume of saline in which it is injected?

in character?

PICKFORD, M. (1956). *In* Modern Views on the Secretion of Urine London: Churchill.
SCHMIDT-NIELSEN, B., SCHMIDT-NIELSEN, K., BROKAW, A., and SCHNEIDERMAN, H. (1948). *J. cell. comp. Physiol.*, **32**, 331.
SHANNON, J. A., and SMITH, H. W. (1935). *J. clin. Invest.*, **14**, 393.
SIROTA, J. H., BALDWIN, D. S , and VILLARREAL, H. (1950). *J. clin. Invest.*, **29**, 187.
SZEGO, C. M., and ROBERTS, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, **61**, 161.
SZEGO, C. M., and ROBERTS, S. (1947). *Endocrinology*, **41**, 322.
VERNEY, E. B. (1947). *Proc. roy. Soc.* B., **135**, 25.
DE WESSELOW, O. L. W. S., and GRIFFITH, W. J. (1934). *Brit. J. exp. Path.*, **15**, 45.

DISCUSSION

Harris: Did I understand correctly that during stimulation in the rat there might be as much as 26 mu.?

Heller: Yes.

Harris: Was that per ml. plasma?

Heller: No, per ml. blood. A total of about 80 mu. in the whole circulation

Harris: Then 60 mu. might be liberated in one short burst from the rat hypophysis?

Heller: Yes, the glands of our animals contain about 800 mu.

[illegible]

is significantly retarded.

Recently we have done some work with the aim of recovering injected vasopressin from the kidneys. However, no antidiuretic activity could

Samuels: Prof. Heller, do you view a low level in the pituitary gland as evidence of high release or of low release into the plasma?

Heller: I think all we can deduce from alterations in the hormone content of the gland is that something has happened. Since, clearly, the hormone content is a result of the amounts of hormone synthesized in or delivered to the gland, and the amounts released from the gland, we cannot say whether a low hormone content means decreased synthesis or increased release.

Samuels: This makes it difficult to interpret pituitary changes physiologically, then, without observations of the changes occurring peripherally at the same time.

Heller: Precisely; I think it stresses Dr Parkes's tenet "back to blood". The physiological situation will be revealed only if the blood level of the hormone is estimated.

of inactivation.

Harris: Yes.

students, that they must not be surprised that hormones are rapidly destroyed in the organism. Hormones could not perform their function otherwise, since their purpose is to adjust to changes in environment and the past must not reflect too much on the present.

that in this situation you have the double problem of changes in hormone release and changes in the hormone concentration in or near the effector cell.

several hours. Dicker and Ginsburg (1950. *Brit. J. Pharmacol.*, 5, 497) failed to find inactivation in rat plasma after 18 hr. at 4°. I think a lot of trouble has been caused by workers who, in similar experiments, did not differentiate between plasma and serum. It is known that proteolytic enzymes are present in serum, and, under the same experimental conditions, one may therefore get rapid inactivation in serum, but not in plasma.

Harris: Prof. Heller, what do you consider are the most promising assay methods for oxytocin?

Heller: The superfused rat uterus seems to be the most sensitive organ for assay. We have, as you know, a good alternative method at our disposal, namely that of estimating the effect of oxytocin in the lactating rabbit.

Harris: The lactating mammary gland of the rabbit is sensitive to about 0 1 mu I was just wondering what sort of a sensitivity you can get with the uterus?

Heller: It is quite as good as that, the superfused uterus responds to 25–50 µu. It is more sensitive to oxytocin than a uterus in an organ bath. However, it seems to us that here as well as in the assay of the antidiuretic hormone, we are now at the end of our tether so far as the sensitivity of the assays is concerned. The next step consists, I think, in extracting the hormones from the blood and in assaying concentrates.

That seems to work; we get reasonably satisfactory results both with the antidiuretic and the oxytocic hormone when we extract with acid ethanol. The recoveries are about 75–80 per cent.

Parkes: Prof. Heller, you showed a couple of diagrams indicating variation in the pressor and oxytocic content of the pituitary during the dioestrus cycle of the rat, which I think you related to the changes that are known to occur during the cycle in the water-content of the animals. What is at the back of all this? Do these changes during the oestrus cycle have some significance, or are they just fortuitous?

Heller: Whether changes in water metabolism come first and affect the posterior pituitary, or whether it is the other way round, is as yet difficult to say. More work on this problem is needed. However, there appear to be, as you pointed out, not only changes in the release of the antidiuretic-pressor principle, but also changes in the release of the oxytocic hormone. This makes the interpretation of our results even more complicated.

Sonenberg: Prof. Heller, do you conclude from your earlier experiments with dialysis that the ADH is bound to proteins?

Heller: I hesitate to draw any conclusions, because this work is not complete. Similar methods to ours have been used to investigate other hormones, for example quite recently by Daughaday (1956. *J. clin. Invest.*, 35, 1428) who concluded that cortisone is partially bound by plasma protein. If one accepts this type of evidence, then our result would also suggest that the neurophysial hormones are partially bound by plasma. But I think that more work must be done to make this conclusion quite convincing.

SOME GENERAL PRINCIPLES IN THE BIOASSAY OF ANTERIOR PITUITARY AND PLACENTAL HORMONES IN BLOOD WITH SPECIAL REFERENCE TO CLINICAL PROBLEMS

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In this paper two main topics will be considered. The first concerns some of the methodological problems involved in the quantitative determination of certain of the anterior pituitary and placental hormones in blood, while the second deals with the blood concentration of these hormones in normal and pathological conditions in man.

It can be definitely stated that the diagnostic value to the clinician of estimations of pituitary and placental hormones in blood is entirely dependent on the reliability of the assay method employed. Accordingly, although this conference is not primarily concerned with methodological problems, I feel it would be appropriate if, before dealing with clinical applications, I outline very briefly some of the difficulties encountered in the assay of these hormones in blood. I shall illustrate my remarks by referring mainly to three hormones. Two of these are elaborated by the anterior pituitary and are the adrenocorticotrophic hormone (ACTH) and the growth hormone; the other, human chorionic gonadotrophin (HCG), is secreted by the chorionic tissue of the placenta.

Difficulties in the Bioassay of Pituitary and Placental Hormones in Human Blood

These fall into five main groups:

1. Problems of sensitivity.
2. Problems of specificity.
3. Problems of precision.

mechanism for sperm ascent. It is a very interesting point because one

Harris: I believe Dr. Barry Cross in Cambridge is interested in that very problem, and was a short time ago studying the effect of oxytocin on motility of the testis. What his results were I do not know.

For the assay of growth hormone in blood the most sensitive test at present available is the so-called "tibial test" which depends on the enlargement of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats. According to Li (1953) this test is not as specific as was once supposed. This worker found that thyroid hormone, TSH and ACTH were all capable of interfering with the response and suggested that, ideally, assays should be conducted in animals which had been subjected to hypophysectomy, adrenalectomy and thyroidectomy.

Problems of Precision

In Table I the precision of some of the assay methods for ACTH is compared in terms of Gaddum's λ. It will be noted

Table I

PRECISION OF SOME ASSAY METHODS FOR ACTH

Method	*Animal*	*Index of precision* (λ)	*Reference*
Sayers test	Hypophysectomized rat	0 176	Sayers, Sayers and Woodbury (1948)
Sayers test	Hypophysectomized rat	0 499	Greenspan *et al.* (1950)
Sayers test	Hypophysectomized rat	0 220	Taylor, Loraine and Robertson (1953)
Sayers test	DCA-treated rat	0 463	Buttle and Hodges (1953)
Adrenal maintenance test	Hypophysectomized rat	0 376	Simpson, Evans and Li (1943)
Thymus weight test	Intact rat	0 316	Bruce, Parkes and Perry (1952)
Eosinophil depression test	Intact mouse	0·330	Speirs (1953)
Corticosteroid production test	*In vitro* assay in rats	0 150	Saffran and Schally (1955)

that the precision of the Sayers test has varied greatly in the hands of different investigators. Sayers, Sayers and Woodbury

4. Problems in relation to existing international standard preparations.
5. Problems associated with the injection of serum or plasma into the test animals.

Problems of Sensitivity

One of the chief difficulties in the estimation of ACTH and growth hormone in blood arises from the fact that available assay methods are not sufficiently sensitive to detect the very small quantities of these hormones present in unextracted serum or plasma. Accordingly, it has been found necessary to concentrate the blood prior to bioassay. In the case of ACTH the most reliable extraction method is probably that depending on oxycellulose adsorption; this procedure has been successfully used by Sayers and his co-workers (Sydnor and Sayers, 1952; Sydnor *et al.*, 1953*a*, *b*). As regards growth hormone, Gemzell, Heijkenskjöld and Ström (1955) have shown that active extracts can be prepared when the blood is concentrated by the technique of Cohn and co-workers (1946). This method depends on fractional alcohol precipitation at low temperatures combined with pH adjustment. It should be emphasized that such extraction methods are usually laborious and tedious and that considerable loss of activity may occur during their performance.

Sensitivity problems do not arise in the case of HCG. This hormone is present in blood in relatively high concentrations and unextracted serum or plasma can generally be injected into the test animals.

Problems of Specificity

For assays of ACTH in blood the Sayers test, due to its high degree of sensitivity, has been the method of choice. This technique was originally described by Sayers, Sayers and Woodbury (1948) and depends on the ability of preparations containing ACTH to cause a depletion in adrenal ascorbic acid in hypophysectomized rats. This assay method is generally believed to be satisfactory on grounds of specificity.

For the assay of growth hormone in blood the most sensitive test at present available is the so-called "tibial test" which depends on the enlargement of the proximal epiphyseal cartilage of the tibia in hypophysectomized rats. According to Li (1953) this test is not as specific as was once supposed. This worker found that thyroid hormone, TSH and ACTH were all capable of interfering with the response and suggested that, ideally, assays should be conducted in animals which had been subjected to hypophysectomy, adrenalectomy and thyroidectomy.

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that the precision of the Sayers test has varied greatly in the hands of different investigators. Sayers, Sayers and Woodbury

(1948) found that the index of precision was generally below 0·2 and concluded that the assay was sufficiently accurate for quantitative work; other investigators, including Greenspan and collaborators (1950) and Buttle and Hodges (1953), have failed to obtain such a high degree of precision and were unable to use the method on a quantitative basis. Morris (1951) has wisely emphasized the importance of the strain of rat in the performance of the Sayers test. Some strains appear to be quite unsuitable due to such factors as insensitivity and great variability of response from one animal to another. Table I also shows that assay methods such as those depending on adrenal weight maintenance in rats, thymus weight in rats and eosinophil depression in mice have a relatively low degree of precision and are not suitable for the quantitative determination of ACTH in body fluids.

In Table II is shown the precision of some assay methods for growth hormone. Of the methods listed only the tibial test

Table II

PRECISION OF SOME ASSAY METHODS FOR GROWTH HORMONE

Method	*Index of precision* (λ)	*Reference*
Plateaued rat weight test	0 198	Marx, Simpson and Evans (1942)
Hypophysectomized rat weight test	0 265	Marx, Simpson and Evans (1942)
Dwarf mice weight test	0 670	Fønss-Bech (1947)
Tail length test in hypophysectomized rats	0·532	Fønss-Bech (1947)
Tibial test in hypophysectomized rats	0 310 0 310	Greenspan *et al.* (1949) Gemzell and Heijkenskjöld (1957)

is sufficiently sensitive to detect the presence of growth-promoting activity in blood. It will be noted that this technique has a relatively large error and is probably not very well adapted to quantitative studies.

The precision of some assay methods for HCG is shown in

Table III. It will be noted that a reasonable degree of precision can be expected with assay methods depending on the

Table III

PRECISION OF SOME ASSAY METHODS FOR *HCG*

Method	*Index of precision* (λ)	*Reference*
Ovarian weight (rat)	0 230	Diczfalusy and Loraine (1955)
Ovarian hyperaemia (rat)	0 450	Albert and Berkson (1951)
Expulsion of spermatozoa (toad)	0 120–0 176	Wohlzogen (1953)
Uterine weight (rat)	0 329	Dorfman and Rubin (1947)
Vaginal smears (rat)	0 276	Loraine (1950*a*)
Total prostatic weight (rat)	0·139	Loraine (1950*a*)
Seminal vesicle weight (rat)	0 200	Watts and Adair (1943)
Total accessory reproductive organs (rat)	0·105	Diczfalusy (1954)

enlargement of the accessory reproductive organs in intact immature male rats and on the expulsion of spermatozoa in amphibia.

Problems in Relation to Existing International Standards

At the present time international standard preparations are available for HCG and for ACTH but not for growth hormone. The international standard for HCG was prepared from pregnancy urine, while that for ACTH was derived from porcine pituitary tissue.

It has been generally assumed that extracts prepared from human blood can be conveniently assayed in terms of standard material prepared from sources other than blood. However, recent work has indicated that this assumption is not necessarily correct. For example, Diczfalusy and Loraine (1955) found that under certain circumstances serum samples of HCG could not be assayed against the standard, while a

similar conclusion was reached by Parrott (1955) in the case of ACTH. Difficulties of a similar nature will almost certainly arise when blood extracts containing thyrotrophic or growth-promoting activity are assayed against the corresponding standards prepared from anterior pituitary tissue. Accordingly, it is essential for investigators to determine by statistical methods whether a given assay procedure which they propose to use for estimations in blood satisfies the recognized criteria of validity. If it does not, it should not be employed in clinical studies.

Problems Associated with the Injection of Serum or Plasma into Test Animals

This section deals entirely with HCG. Many investigators throughout the years have estimated the serum concentration of this hormone in normal and abnormal pregnancy, and in the course of these studies a large number of different bioassay techniques have been employed. Recently it has become apparent that in such estimations on unextracted serum the end-point of the test must be selected with care in order to obtain valid results. This information has been obtained by comparing the potency of HCG dissolved in saline with that of HCG dissolved in serum and plasma. By making such a comparison it is possible to divide assay methods for the hormone into three groups.

(*a*) *Tests in which the figure of* 100 *per cent is within the fiducial range.* This group includes methods depending on the enlargement of the various accessory reproductive organs in intact and hypophysectomized immature male rats. Such tests provide a true estimate of potency and can be used with confidence for the quantitative determination of HCG in human serum.

(*b*) *Tests in which the figure of* 100 *per cent is without the fiducial range.* Among such tests are those depending on uterine weight in intact immature rats and on expulsion of spermatozoa in amphibia. These methods provide either an overestimate or an underestimate of the true potency and are unsuitable for use in the clinical field.

(c) *Tests in which the fiducial range is dependent on the concentration of serum injected.* The concentration of the serum is of importance when the ovarian hyperaemia test in rats is used. This method yields valid results only if the serum is diluted prior to injection (Albert and Berkson, 1951; Borth, Lunenfeld and de Watteville, 1957).

Some of the results obtained with the various assay methods are shown in Table IV.

Table IV

COMPARISON OF THE POTENCY OF HCG DISSOLVED IN SALINE WITH THAT OF HCG DISSOLVED IN SERUM OR PLASMA USING VARIOUS ASSAY METHODS* (AFTER BORTH, LUNENFELD AND DE WATTEVILLE, 1957)

Method	*Conc of serum % v/v*	*Mean potency ratio %*	*Fiducial limits %* ($P = 0.95$)	*Reference*
Total prostatic weight (intact rats)	100	106	84–132	Diczfalusy and Loraine (1955)
Seminal vesicle weight (intact rats)	100	114	80–199	Diczfalusy and Loraine (1955)
Ventral prostatic weight (hypophysectomized rats)	100	117	75–192	Diczfalusy and Loraine (1955)
Uterine weight (intact rats)	100	190	160–230	Diczfalusy and Loraine (1955)
Expulsion of spermatozoa (*Rana esculenta*)	50	30	10–60	
	100	20	7–30	Salvatierra and Torres (1952)
Expulsion of spermatozoa (*Bufo viridis*)	100	170	150–190	Lunenfeld *et al* (1957)
Ovarian hyperaemia (intact rats)	10	100	Not stated	
	25–100	Decreasing to 30% as serum conc. increased	Not stated	Albert and Berkson (1951)
	35	90	60–100	
	70	100	90–110	Borth, Lunenfeld and de Watteville (1957)
	100	70	50–90	

* The potency of HCG in 0 9 per cent saline is assumed to be 100 per cent.

The Clinical Application of Assays of ACTH in Blood

Normal Subjects

In most determinations conducted on human blood the relatively sensitive Sayers test has been the bioassay method of choice. There has been considerable disagreement among various investigators regarding the normal levels of blood ACTH in man. The available evidence has recently been critically examined by Sayers (1955) and the present account is based largely on his conclusions.

The most careful and detailed studies on the ACTH content of normal blood have been made by workers at the Mayo Clinic and at Cleveland under the leadership of Albert and Sayers respectively. In 1949 Taylor, Albert and Sprague, using unextracted serum, were unable to demonstrate corticotrophic activity in the blood of normal individuals. Subsequently Paris and co-workers (1954) extended these observations by employing the oxycellulose extraction method and found that no significant depletion in adrenal ascorbic acid occurred when concentrates equivalent to 40 ml. whole blood were administered to hypophysectomized rats. Sydnor and co-workers (1953*b*) and Paris and co-workers (1954) have concluded that the concentration of ACTH in the blood of normal subjects is very low indeed, being usually less than 0·5 mu./100 ml. whole blood (mu. = milliunits).

These results are in marked contrast to those reported by Bornstein and his co-workers (Bornstein and Trewhella, 1950; Parrott, 1951; Bornstein, Gray and Parrott, 1952) who claimed that ACTH was present in normal blood in the very high concentration of 30 to 200 mu./100 ml. These investi-

high blood levels of ACTH in normal subjects should be viewed with scepticism. It is almost certain that, if the blood concentration of ACTH were in the range reported by

Bornstein and his co-workers, such levels would be associated with the clinical features of adrenocortical hyperfunction.

Pathological Conditions

(a) *Adrenal Hypofunction.* All investigators agree that in cases of untreated Addison's disease the ACTH titre in blood is increased. Taylor, Albert and Sprague (1949) were the first to detect activity in the serum of patients with this disease and Sayers (1956, personal communication) has reported concentrations of approximately 2–4 mu./100 ml. Treatment of such patients with cortisone causes a decrease in blood ACTH levels and soon after the onset of such therapy activity can no longer be detected in peripheral blood.

According to Sayers (1955) the blood ACTH concentration rises after bilateral adrenalectomy. Further observations in patients treated in this way will be awaited with interest.

(b) *Adrenal Hyperfunction.* Sydnor and co-workers (1953a) found abnormally high titres in cases of congenital adrenal hyperplasia, but Taylor, Albert and Sprague (1949) were unable to detect any activity in serum of patients with Cushing's syndrome.

(c) *"Stress" Conditions.* Under such circumstances elevated blood levels of ACTH are by no means invariable. Sayers (1955) found that the administration of concentrates equivalent to 20 to 40 ml. blood obtained from two patients with miliary tuberculosis did not produce any significant depletion of adrenal ascorbic acid in hypophysectomized rats. Negative results were also found in healthy male subjects exposed to excessive heat and to abnormally low environmental temperatures.

It must be emphasized that present methods for the determination of ACTH in blood are quite unsuitable for routine use in the clinical field. Such techniques are expensive and laborious, and frequently large volumes of blood are required for individual estimations. Nevertheless, in centres in which facilities are adequate, valuable clinical information can sometimes be obtained in selected cases by the assay of this

hormone in blood. For further discussion regarding the clinical application of ACTH assays the reader is referred to a recent review of the subject (Loraine, 1957).

The Clinical Application of Assays of Growth Hormone in Blood

Growth hormone has not yet been detected in the blood of normal individuals, but growth-promoting activity has been found in the plasma of a small number of patients with gigantism and acromegaly (Kinsell *et al.*, 1948; Gemzell, Heijkenskjöld and Ström, 1955). Recently Gemzell, Heijkenskjöld and Ström (1955), using the tibial test in hypophysectomized rats, have succeeded in demonstrating the presence of the hormone in lyophilized plasma from pooled retroplacental blood and in plasma from umbilical cord blood.

One of the most urgent problems in present day endocrinology is the development of a reliable assay method for the quantitative determination of growth hormone in body fluids. If such a technique were available it would have a wide application in clinical research.

The Clinical Application of Assays of Serum HCG

This subject has been recently reviewed (Loraine, 1956, 1957) and will not be considered in detail in the present paper. It should be emphasized that assay results must now be expressed in terms of the international standard and not in arbitrary "animal" units.

Normal Subjects

In Fig. 1 are shown the mean urinary and serum levels of HCG throughout normal pregnancy. It will be noted that very large amounts of HCG appear in blood and urine during the first trimester of normal pregnancy. This constitutes the so-called "peak period". Readings made at this time are very variable from one subject to another and may range from

20,000 to 100,000 i.u. per 24 hours of urine or per litre of serum. The time at which the HCG levels begin to decrease is also very variable but usually the peak period has passed by the 15th week of pregnancy. In the second and third trimesters the figures for both serum and urine are much more constant than in early pregnancy. Loraine (1950*a*, 1957)

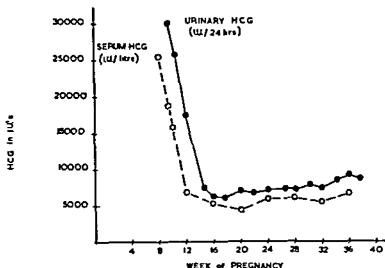


FIG. 1. Mean estimations of urinary and serum HCG throughout normal pregnancy (after Loraine, 1957).
(Method of assay = prostatic weight in rats.
Total number of estimations—urine 136 : serum 140.)

calculated that at this time they lay in the range 4,000 to 11,000 i.u. ($P = 0{\cdot}99$) and suggested that readings consistently outside this range should be regarded as pathological. Fig. 1 illustrates the very close quantitative relationship between urine and serum levels of HCG. At the present time HCG is the only hormone which can be estimated with reasonable accuracy in both blood and urine and of which reliable calculations can be made of the renal clearance in health and disease (Gastineau, Albert and Randall, 1949; Loraine, 1950*b*).

Pathological Conditions

Abnormally high serum HCG levels have been encounte in the majority of cases of hydatidiform mole and of chori epithelioma of the uterus and of the testis. For further infor ation on the diagnostic value of HCG readings in these con tions the reader is referred to articles by Hamburger (19 1946), Twombly (1944) and Hobson (1955).

(*a*) *Pre-eclamptic Toxaemia.* The serum concentration HCG in toxaemia of pregnancy has been studied by vari investigators including Smith and Smith (1934, 1948), Tay and Scadron (1939) and Loraine and Matthew (1950). In t experience of Loraine and Matthew (1950) severe cases pre-eclamptic toxaemia showed a mean serum HCG conc tration which was significantly higher than that found moderate and mild cases. These workers were unable correlate the high HCG readings on the one hand with a clinical feature such as the presence of oedema, the deg of albuminuria and the height of the blood pressure on t other. They concluded that at the present time rout assays of serum HCG would not be of much assistance the clinician in the management of cases of pre-eclamp toxaemia.

(*b*) *Diabetic Pregnancy.* The diagnostic and prognos value of serum HCG determinations has been investigated White and her collaborators (White, 1952; White and Hu 1943), Keltz, Keaty and Hellbaum (1950) and Loraine a Matthew (1954). Only in the paper of Loraine and Matth (1954) were the assay results expressed in terms of the int national standard. Loraine and Matthew (1954) demonstrat that approximately 30 per cent of pregnant diabetic subje showed abnormally high readings of serum HCG, but we unable to correlate the HCG levels on the one hand with a of the medical or obstetrical findings on the other. The workers concluded that serum HCG estimations in diabe pregnancy were of little or no value from the prognostic poi of view.

Summary

Some of the problems associated with the quantitative determination of ACTH and growth hormone in blood are discussed. Difficulties arise because of the lack of methods sufficiently sensitive to detect the very small quantities of these hormones present in blood, because of the low degree of precision of some of the assay methods proposed, and because of the necessity of estimating blood extracts in terms of standards prepared from anterior pituitary tissue. In the case of HCG care is necessary in the selection of an assay method for estimation in serum in order to avoid either an overestimate or an underestimate of potency.

The diagnostic value of estimations of ACTH, growth hormone and HCG in blood is briefly reviewed. It is emphasized that, at present, methods for the determination of ACTH and growth hormone in blood are quite unsuitable for routine use in the clinical field.

REFERENCES

ALBERT, A., and BERKSON, J. (1951). *J. clin. Endocrin. Metab.*, **11**, 805.
BORNSTEIN, J., and TREWHELLA, P (1950). *Lancet*, **2**, 678.
BORNSTEIN, J., GRAY, C. H , and PARROTT, D. M. V. (1952). *J. Endocrin.*, **8**, 40.
BORTH, R., LUNENFELD, B , and DE WATTEVILLE, H. (1957). *Acta endocr., Copenhagen*, **24**, 119.
BRUCE, H. M , PARKES, A. S , and PERRY, W. L. M. (1952). *Lancet*, **1**, 790.
BUTTLE, G. A. H., and HODGES, J. R (1953). Ciba Foundation Colloquia on Endocrinology, **5**, 147. London: Churchill.
COHN, E. J., STRONG, L E , HUGHES, W. L Jr., MULFORD, D. J., ASHWORTH, J. N , MELIN, M , and TAYLOR, H. L. (1946) *J. Amer. chem. Soc.*, **68**–1, 459.
DICZFALUSY, E. (1954). *Acta Endocr., Copenhagen*, **17**, 58.
DICZFALUSY, E., and LORAINE, J. A. (1955). *J clin. Endocrin. Metab.*, **15**, 424.
DORFMAN, R. I., and RUBIN, B. L (1947) *Endocrinology*, **41**, 456.
FØNSS-BECH, P. (1947) *Acta pharm. tox* , **3**, suppl. 3.
GASTINEAU, C. F., ALBERT, A , and RANDALL, L. M. (1949) *J. clin. Endocrin.*, **9**, 615.
GEMZELL, C. A., and HEIJKENSKJOLD, F. (1957). *Acta endocr., Copenhagen.* In press.

GEMZELL, C. A., HEIJKENSKJÖLD, F., and STROM, L. (1955). *J. Endocrin. Metab.*, 15, 537.
GREENSPAN, F. S., LI, C. H., SIMPSON, M. E., and EVANS, H. M. (19 *Endocrinology*, 45, 455.
GREENSPAN, F. S., LI, C. H., SIMPSON, M. E., and EVANS, H. M. (19 *In* Hormone Assay, p. 273. Ed. C. W. Emmens. New Y Academic Press Inc.
HAMBURGER, C. (1944). *Acta obstet. gynec. scand*, 24, 45.
HAMBURGER, C. (1946). *J. Endocrin.*, 5, proc. xxiv.
HOBSON, B. M. (1955). *J. Obstet Gynaec*, *Brit. Emp.*, 62, 354.
KELTZ, B F., KEATY, C., and HELLBAUM, A A. (1950). *Southern J.*, 43, 803.
KINSELL, L. W., MICHAELS, G. D., LI, C. H., and LARSEN, W. E. (19 *J. clin. Endocrin*, 8, 1013.
LI, C. H. (1953). Ciba Foundation Colloquia on Endocrinology, 5, London: Churchill.
LORAINE, J. A. (1950*a*). *J. Endocrin.*, 6, 319.
LORAINE, J. A. (1950*b*). *Quart. J. exp. Physiol.*, 36, 11.
LORAINE, J. A. (1956). *Vitam. & Horm.*, 14, 305.
LORAINE, J. A. (1957). The Clinical Application of Hormone As *Chapter 5. Edinburgh: Livingstone. In press.*
LORAINE, J. A., and MATTHEW, G. D. (1950). *J. Obstet. Gynaec., Emp.*, 57, 542.
LORAINE, J. A., and MATTHEW, G. D. (1954). *Le Diabète*, 2, 43.
LUNENFELD, B., RABAU, E, HARELL-STEINBERG, A., and SZEJNBE A. (1957). *Acta endocr., Copenhagen*, 24, 113
MARX, W., SIMPSON, M. E., and EVANS, H M. (1942) *Endocrinol* 30, 1
MORRIS, C. J. O. R (1951). *Analyst*, 76, 470

SALVATIERRA, V., and TORRES, A. (1952). *Acta ginec., Madrid*, 111, 4
SAYERS, G. (1955). *J clin. Endocrin. Metab.*, 15, 754.
SAYERS, M. A., SAYERS, G, and WOODBURY, L. A. (1948). *Endocr ology*, 42, 379.
SIMPSON, M. E., EVANS, H. M., and LI, C. H. (1943). *Endocrinology*, 261.
SMITH, G. V., and SMITH, O W. (1934). *Amer. J. Physiol.*, 107, 128.
SMITH, G. V., and SMITH, O. W. (1948). *Physiol Rev.*, 28, 1.

SAYER
Y., 7
402.
SYDNOR, K. L., SAYERS, G, BROWN, H., and TYLER, F. H. (1953*b*). *clin. Endocrin. Metab.*, 13, 891.

TAYLOR, H C. Jr., and SCADRON, E. N. (1939). *Amer. J. Obstet. Gynec.*, 37, 963.
TAYLOR, A. B., ALBERT, A., and SPRAGUE, R. G. (1949). *Endocrinology*, 45, 335.
TAYLOR, N. R. W., LORAINE, J. A., and ROBERTSON, H. A. (1953). *J. Endocrin.*, 9, 334.
TWOMBLY, G. H. (1944). *Surgery*, 16, 181.
[illegible] *Gynec.*, 46, 183.
[illegible] edit., ed.
[illegible]
WOHLZOGEN, F. X. (1953). *Arch. exp. Path. Pharmak.*, 217, 482.

DISCUSSION

[illegible]

between levels found in blood samples, taken at various hours of the same day. Fiducial limits and pooled indices of precision have not yet [illegible]

[illegible]

useful to characterize the precision of a certain method as it has been obtained in the hands of a certain investigator. The λ value does not characterize the method alone as such, but strains of animals and [illegible]

[illegible]

other methods.

Borth: Oh, yes, I agree Obviously such comparisons would not be possible without a common yardstick for the precision, such as λ or *L*.

Heller: You mentioned the renal clearance of HCG. Did you say that it was not influenced by pathological conditions?

Loraine: We found that in normal pregnancy the mean renal clearance of HCG was less than 1·0 ml. per minute. Constant figures for

depend to a very large extent on the clinical criteria used in classifying the toxaemic patients.

Heller: How is it cleared? By what mechanism is it excreted?

Loraine: At present we do not know the mechanism of HCG clearance. The figures we obtained were relatively low and would be typical of protein clearance

Randle: Dr. Loraine, have you tried using human growth hormone or extracts of human pituitaries as standards for the assay of growth hormone activity in human plasma? This may be of importance because of the species differences in growth hormone which have been reported.

I should like to ask Dr. Loraine what he thinks also of Segaloff's values for the growth hormone activity of normal human plasma (Segaloff, A. (1955). *In* The Hypophyseal Growth Hormone, Nature and Actions, p. 53. New York: McGraw-Hill). He finds as much as 30 μg. growth hormone per ml. normal human plasma by the tibia test. If these values do reflect the growth hormone content of normal human plasma, then it should be possible to demonstrate an increase

Proc., 15, 39).

There is some experimental evidence to suggest that growth hormone in blood may be a different molecule from that which is extracted from the pituitary gland. Thus although growth hormone *in vivo* can inhibit the utilization of glucose by muscle, no such action of the hormone has been reliably demonstrated *in vitro* Bornstein and Park (1953. *J. biol. Chem*, 205, 503) have obtained evidence which suggests that an inhibitor of glucose uptake may appear in the plasma of rats under the influence of growth hormone and cortisone. This raises another problem in relation to the assay of growth hormone in blood.

Loraine: These are very interesting questions, Dr. Randle, but I doubt very much if I can give satisfactory answers to them. We have

that, as in the case of ACTH, care is necessary in the selection of an assay method to be used in clinical studies. I am afraid I cannot offer any comment on your question about the effect of growth hormone on carbohydrate metabolism.

Parkes: How long a course of injection does the growth hormone assay involve?

Loraine: The time taken will depend on the method; it is usually a fairly lengthy procedure In assays involving "plateaued" rats the injection period extends over 15 to 20 days. With the hypophysectomized rat weight test the animals are rested for 10 to 12 days post-operatively, the injection period varies from 10 to 15 days. With the tibial test in hypophysectomized rats the injection period is only 4 days.

Parkes: Not enough to immunize the animals then?

Loraine: No, I do not think so.

Prunty: One of the problems coming into the question seems to be what happens to the hormone when you put it into the blood? Are its action and potency modified in any way?

Borth. We have found that in certain experiments with ACTH if you add some samples of serum you get quite a significantly different slope in your assay from just a pure preparation.

ments were done for ACTH, and comparisons made between gland extracts and "pure" solutions. But there still arises the question of whether the recovery experiments, with these different methods of

unless the correctly designed recovery experiments were done. Could you give us some details on that?

Loraine. Regarding the question of recovery experiments with the oxycellulose method, I think that most investigators have obtained

high levels which he reported

Farrell With respect to the recovery experiments, those were done

in the original work of Sydnor and Sayers (1952 *Proc Soc. exp. Biol Med.*, 79, 432). They compared ACTH content, as determined by direct cross-transfusion of blood, with the oxycellulose technique and they checked very nicely.

Loraine· On the question of the extraction of growth hormone from blood the recoveries reported by Gemzell and his collaborators were rather low, being of the order of 40–50 per cent.

Bush: I still don't see that we have any scientific reason explaining why Bornstein and Trewhella find results of 200–300 mu /100 ml, whereas the method which I suspect is more correct, the oxycellulose method, gives results of 0 1 and 0 2; and I don't think one can just leave this very large difference in results lying around unexplained. One would like to know the reason, because big mistakes are sometimes the way in which significant facts are discovered.

Loraine: I think Dr. Sayers has made quite a number of comparisons ... He came

good method

Parkes· I don't quite understand your worry, Bush.

Bush: I feel that in this question of biological assay, if you are going to take an animal and do a certain number of standard procedures with it and you are then going to inject something, there should be an explanation found as to why one observer gets a result which is so grossly different from that of another.

I think it is a messy situation when we have a 200- to 2000-fold ... definite difference

Trewhella have never been adequately confirmed

Roberts. Is the situation really so simple? I don't wish to cast any doubt on the results obtained by Dr. Sayers and his group, partly because we have obtained similar results under a variety of conditions, but nonetheless several other laboratories have reported high values for blood ACTH.

Heller: So far as the posterior pituitary principles are concerned, the lower values have, historically speaking, always been the correct ones, and this may apply to other hormones. One is, I think, a little influenced by that. It does not of course exclude the possiblity that for once the higher values may be the right ones.

Randle: I believe Bornstein and his colleagues did not make 4-point assays of ACTH activity in plasma extracts. Four-point assays were

later reported by Parrott (Parrott, D. M. V. (1955). *J. Endocrin.*, **12**, 120) and were for the most part unsatisfactory in that the slopes for plasma extracts and ACTH were different. If the high values which have been reported for plasma ACTH activity are correct, but do not reflect the corticotrophin content of plasma, then either plasma contains substances which may potentiate the action of corticotrophin with adrenal ascorbic acid depleting activity. The latter seems unlikely in view of high specificity of the Sayers test.

Loraine: Dr. Parrott attempted to conduct 4-point assays but many of these were invalid. There were occasional times when high activity was found. We have found no real explanation for this.

I believe that most people believe that the Sayers test is reasonably satisfactory on grounds of specificity. I suppose, however, that this conclusion may not necessarily be justifiable.

[illegible]

activity appears.

Roberts: I might say that this is an important problem right now, because methods of assay for ACTH other than the Sayers procedure have been coming into greater and greater popularity. I hope to say a few words about this to-morrow This same question will come up then, namely, which assay method, if any, gives the correct values for blood ACTH.

Loraine: The technique you will describe is an *in vitro* method?

Roberts: Yes, in part. Different results may be obtained by all three assay procedures, that is, ascorbic acid decline, and *in vitro* and *in vivo* release of corticosteroids Which one is correct, or whether they are all correct under different circumstances, is impossible to decide at this time.

INHIBITION OF THYROTROPHIC ACTIVITY WITH ACETYLATED THYROTROPHIC HORMONE PREPARATIONS*

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IT was previously reported (Sonenberg and Money, 1955) that the preparation of a radiosulphur, ^{35}S, labelled pituitary preparation containing thyrotrophic activity resulted in the loss of the normal stimulatory activity without preventing the localization of ^{35}S in the thyroid. This observation suggested that the biological activity of a trophic factor like TSH might contain both a localizing and a stimulatory property. If such were the case, the latter might be lost with retention of the former. With this as a working hypothesis, derivatives of TSH preparations were prepared in order to determine if non-stimulatory trophic hormone preparations could be used as inhibitors of thyrotrophic hormone activity. Of the many preparations tested only two derivatives, both involving known substitutions on the amino group, gave promise of possessing inhibitory activity for exogenous and endogenous TSH. Earlier publications (Sonenberg and Money, 1956, 1957) reported the results of studies with one such derivative, acetylated thyrotrophic hormone preparation.

In this paper we shall present (1) the results of experiments concerned with the possible mechanism of inhibitory action of derivatives of TSH preparations and (2) further studies with acetylated TSH preparations as well as other acetylated substances.

Methods

Chemical

The techniques employed for the preparation of derivatives of thyrotrophic hormone preparations have previously been reported for the ^{35}S sulphate ester (Sonenberg and Money, 1955) and for the acetylated derivatives (Sonenberg and Money, 1957).

Acetylated bovine serum albumin was prepared by a technique similar to that employed with pituitary preparations.

Biological

For the evaluation of thyrotrophic and gonadotrophic stimulatory and inhibitory activity, the techniques employed in chicks* were the same as those reported earlier (Sonenberg and Money, 1957). Control substances were similarly administered to evaluate their effect on thyrotrophic and gonadotrophic activity. Prior to autopsy all animals were given 0·1 μc ^{131}I subcutaneously. The interval of time ^{131}I was administered preceding killing varied depending on the exact nature of the study being done. These times are indicated below where the individual experiments are discussed. Following sacrifice, the thyroids of animals receiving ^{131}I were processed as previously described (Sonenberg and Money, 1957).

The data have been evaluated according to the method of Fisher (1941). In this paper significant observations are considered those with P values of 0·05 or less.

Results

Influence of carrier on tissue concentration of ^{35}S following administration of labelled pituitary preparations

The effect of increasing amounts of carrier TSH on the concentration of radioactive sulphur in the thyroid, muscle

* We would like to express our appreciation to Mr. Monroe Babcock of the Babcock Poultry Farm, Inc , Ithaca, New York, for the generous supply of white Leghorn cockerels used in these studies.

and blood of chicks at various intervals after the injection of an ^{35}S ester pituitary preparation is summarized in Table I. The ^{35}S-labelled pituitary preparation used contained 0·1 mg. protein and 100 μc radioactivity. For carrier purposes 1 and 10 mg. unlabelled material were given simultaneously with the labelled preparation. Injections, after mixing of the sample, were made as a single intravenous administration through a wing vein. The animals were sacrificed by decapitation and exsanguination 1·5, 5 and 24 hours later. Representative tissues were removed, weighed and assayed for radioactivity. The results in Table I have been expressed as the

Table I

EFFECT OF INCREASING AMOUNTS OF CARRIER AP ON ^{35}S LOCALIZATION WHEN SIMULTANEOUSLY INJECTED WITH ^{35}S-LABELLED PITUITARY PREPARATION (TSH ^{35}S No. 32)

Time after ^{35}S preparation hr.	*Carrier mg*	*Per cent ^{35}S per g.*		
		Thyroid	*Muscle*	*Blood*
1.5	0·1	0·541	0·011	0·073
1.5	1·0	0·498	0·016	0·069
1.5	10·0	0·300*	0·030	0·089
5	0·1	0·419	0·014	0·054
5	1·0	0·480**	0·019	0·066
5	10·0	0·228*	0·021	0·080*
24	0·1	0·410	0·014	0·024
24	1·0	0·346*	0·010	0·019
24	10·0	0·202*	0·010	0·028

For statistical evaluation the groups receiving 1·0 mg and 10·0 mg of carrier pituitary preparation (AP) were compared to the group receiving 0·1 mg of protein at the corresponding times.

* — indicates a P value of 0·01 or less,
** — indicates a P value between 0·05 and 0·01

fraction of the administered dose of ^{35}S per g. tissue. As may be seen in Table I, at the times indicated there was less ^{35}S in the thyroids of animals receiving 10 mg. of the carrier preparation simultaneously with the ^{35}S-labelled pituitary

preparation than in the thyroids of chicks receiving only the ^{35}S-labelled pituitary preparation. At these same times, however, there was no similar decrease of ^{35}S in muscle, blood or many other tissues which were examined but have been omitted from the Table. At 5 hours there was actually a significant increase in the concentration of ^{35}S in blood when the labelled preparation was administered with 10 mg. of carrier thyrotrophin preparation.

Frequency of administration of acetylated pituitary preparation and the response to unmodified material

Table IIA shows the effect of unmodified pituitary preparation administered daily for 6 days, while the acetylated material was administered for different periods of time during this interval. Each animal, however, received the same total dose of acetylated pituitary preparation regardless of the number of days the animals were injected. As may be seen from the data the average thyroid weight for uninjected chicks was 2·8 mg. The average thyroid weight in chicks receiving the unmodified pituitary preparation was 7·4 mg. Simultaneous injections of acetylated pituitary preparation for the first 2 or 4 days and unmodified thyrotrophin for 6 days gave no significant reductions in the thyroid weights compared to animals receiving only the unmodified preparation. A significant decrease of the thyroid weight from 7·4 mg. to 5·4 mg. was observed when the acetylated pituitary preparation, as well as the unmodified pituitary preparation, was given for 6 days. With these injection schedules and dose of pituitary derivative there was slight but significant inhibition of the increased ^{131}I uptake in the thyroid only in the group injected for 2 days with acetylated pituitary preparation. There actually was a significant increase from 24 6 mg. in the testes weight of the groups treated for 4 days with acetylated thyrotrophin to 30·2 mg. The results of experiments when the same total dose of acetylated pituitary preparation was administered during the last 2, 4 or all 6 days, while the unmodified pituitary preparation was given daily for 6 days, are

Table II

EFFECT OF VARYING THE TIME OF MULTIPLE INJECTIONS OF ACETYLATED PITUITARY PREPARATION OVER 6-DAY PERIOD

Treatment				*Chick weight g.*	*Thyroid weight mg.*	*% ¹³¹I Thyroid*	*Testes weight mg.*
Subst.	*Days*	*Subst.*	*Days*				
IIA.							
TI 90	1–2	AP	1–6	60	6·3	8·3**	31·1
TI 90	1–4	AP	1–6	64	7·6	10 4	30 2**
TI 90	1–6	AP	1–6	63	5 4*	8·9	26·6
—	—	AP	1–6	59	7 4*	10 3*	24·6*
No injections				57	2 8	0 7	13 5
IIB:							
TI 86	5–6	AP	1–6	64	6 5	11 6	28 4
TI 86	3–6	AP	1–6	63	6·7	12·5	30 5**
TI 86	1–6	AP	1–6	60	5 2*	12 2	20 5*
—	—	AP	1–6	64	7 2*	11 5*	25 3*
No injections				62	2 7	1·1	9·7

Unmodified pituitary preparation (PD × 6585) was given daily to each [illegible]

preparations.

For statistical evaluation the groups receiving the acetylated pituitary preparations (TI 90 or TI 86) and the unmodified pituitary preparation (*AP*) were compared to the group treated with unmodified pituitary preparation (AP) alone.

* — indicates a *P* value of 0 01 or less,
** — indicates a *P* value between 0 05 and 0 01.

summarized in Table IIB. The thyroid weight was 2·7 mg. in the uninjected animals and 7·2 mg. in the unmodified pituitary-treated group. The thyroid weights were slightly but not significantly decreased in the groups receiving the acetylated thyrotrophin for the last 2 and 4 days and the unmodified thyrotrophin preparation for 6 days. The thyroid weights were significantly reduced from 7·2 mg. in the animals injected only with unmodified thyrotrophin to 5·2 mg. in the animals receiving the unmodified pituitary preparation as well

as the acetylated pituitary preparation for the entire 6-day period. With the injection of this acetylated pituitary preparation (TI 86) and unmodified pituitary preparation there was no inhibition of increased thyroidal radioiodine concentration compared to the animals receiving only the unmodified preparation. In chicks treated with TI 86 and unmodified material for 6 days, there was a significant decrease in testes weight which was, however, not statistically significant when related to body weight. There actually was a slight increase in testes weight in animals treated with the acetylated pituitary preparation (TI 86) and unmodified material for the last 4 days.

Effect of varying time of administration of acetylated pituitary preparation on thyroidal radioiodine concentration

These experiments were designed to assess the interval of antagonism of acetylated TSH preparations for endogenous thyrotrophin. In order to obtain an increased concentration of radioactive iodine in the thyroids so that a decrease associated with treatment with acetylated pituitary preparations would be more apparent, chicks were maintained on a low iodine diet for three days from the first day of life. To determine the time of maximum concentration of ^{131}I in the thyroids, groups of animals were given a tracer dose of 0·1 μc ^{131}I and were sacrificed at various periods of time from 3 to 24 hours later (Table IIIA). Other animals received an injection of 15 mg. acetylated pituitary preparation at various times either before or after the radioiodine. All chicks in this latter study (Table IIIB) were sacrificed 24 hours after the tracer dose of ^{131}I.

The groups of chicks that received a tracer dose of radioiodine and no acetylated pituitary preparation demonstrated a maximum thyroid concentration of 21·7 per cent at 3 hours with a gradual decrease to 7·8 per cent at 24 hours (Table IIIA). The animals receiving only the low iodine diet and sacrificed 24 hours after the ^{131}I had a thyroid uptake of 7·8

Table III

EFFECT OF VARYING THE INTERVAL OF TIME BETWEEN A SINGLE INJECTION (15 MG.) OF AN ACETYLATED PITUITARY PREPARATION (TI 112) AND A TRACER DOSE OF RADIOIODINE

IIIA	Sacrifice time after tracer dose of ^{131}I†	% ^{131}I/Thyroid
	3	21·7*
	6	19·4*
	8	19·3*
	12	16 0*
	15	11·7**
	18	10·1
	24	7 8

† These chicks received no acetylated pituitary preparation

IIIB	Hours before or after ^{131}I‡	% ^{131}I/Thyroid
	−18	17·4**
	−15	17·9*
	−12	13·6**
	− 8	12·7*
	− 6	18·5*
	− 3	14·5*
	+ 3	11·4
	+ 6	10·1
	+ 8	8·9
	+12	9 6
	+15	9 2
	No treatment	7·8

‡ The chicks were sacrificed 24 hours after the injection of radioiodine

For statistical evaluation all groups, whether receiving the acetylated pituitary preparation or not, were compared to the group sacrificed at 24 hr. without treatment.

* — indicates a P value of 0 01 or less,
** — indicates a P value between 0 05 and 0 01.

per cent (Table IIIB). When a single injection of 15 mg. acetylated pituitary preparation was administered 3 to 18 hours prior to the radioiodine the uptake was significantly increased to a range between 12·7 and 18·5 per cent. However, if the same dose of acetylated pituitary preparation was given 3 to 15 hours after the ^{131}I, less radioactivity was concentrated in the thyroid than with prior treatment. As may be seen from Table IIIB the concentration of thyroidal ^{131}I was slightly increased, but not to a statistically significant extent, from that found in control animals which had received no acetylated material.

Effect of non-pituitary acetylated substances on thyrotrophic and gonadotrophic activity

In order to ascertain whether the inhibition of thyrotrophic activity was a property of any acetylated protein or an acetylated amino acid or merely the acetyl group *per se*, other acetylated substances were tested in a similar manner (Sonenberg and Money, 1957) for the possibility of inhibiting exogenous or endogenous thyrotrophic or gonadotrophic activity.

The increase in testes weight, thyroid weight or uptake of ^{131}I induced by a pituitary preparation with gonadotrophic and thyrotrophic activity was not altered by the simultaneous injection of several acetylated substances in molar doses approximately 100 times greater than the acetylated pituitary preparation. These included another acetylated protein, bovine serum albumin (Sonenberg and Money, 1957), as well as acetic acid alone (Sonenberg and Money, 1957). In addition, neither acetyl-L-tryptophan, acetyl-DL-alanine, acetyl-DL-valine, acetyl-L-tyrosine, nor acetyl-L-leucine had any effect on thyrotrophic or gonadotrophic activity. There was no alteration in testes and thyroid weight or radioiodine uptake of chicks receiving the acetylated amino acids without the unmodified pituitary preparation.

Discussion

The specificity of the localized radioactive sulphur in the thyroids of chicks receiving an ^{35}S ester pituitary preparation is suggested by the large thyroid/blood ratios of 8 to 20 as contrasted with ratios of less than 0·5 for muscle/blood (Table I). It has previously been demonstrated (Sonenberg and Money, 1955) that the administration of ^{35}S inorganic sulphate with an unmodified pituitary preparation failed to lead to significant concentrations of radioactive sulphur in the thyroid. With this in mind, the decrease in localization of radioactive sulphur in the thyroid as increasing amounts of thyrotrophic hormone preparations are simultaneously administered (Table

I), suggests that the non-radioactive molecules compete with the ^{35}S-labelled molecules for localization in the thyroid. This would indicate that there is a limited number of binding sites in the thyroid, presumably for thyrotrophin, so that one could conceivably saturate the gland with non-stimulatory but localizing molecules of thyrotrophin (Sonenberg and Money, 1957). This would form the basis for using a non-stimulatory but localizing derivative of a pituitary preparation to antagonize exogenously administered or endogenously produced trophic hormone.

The specificity of an acetylated thyrotrophic hormone preparation as an inhibitor of thyrotrophin is supported by the observations that neither acetylated bovine serum albumin nor acetic acid nor acetylated amino acids have a similar inhibitory effect for thyrotrophin even when administered in molar ratios 100 times greater than the acetylated pituitary preparation. (The original pituitary preparation was assumed to have a molecular weight of 10,000 despite its known heterogeneity.)

The data (Table IIA and IIB) suggest that an acetylated pituitary preparation must be administered daily for 6 days in order to antagonize partially the thyroid response to an unmodified pituitary preparation injected similarly over the same period of time. As previously reported (Sonenberg and Money, 1957) approximately 4 to 25 times more of the derivative than the pituitary preparation must be administered to offset partially the action of the latter. When the thyrotrophin was administered daily for 6 days, the acetylated pituitary preparation, in the same total dosage over the first or last 2 or 4 days, did not significantly modify the thyroid weight response to thyrotrophin preparations. Although these data suggest that the acetylated pituitary preparation must act over the same period of time as unmodified thyrotrophin they give no indication how long the effect of any one injection persists. They also imply that the block to thyrotrophic hormone action is incomplete, for injecting an acetylated pituitary preparation for 2 or 4 days in the same total dosage

as in the 6-day experiment did not significantly reduce the response to unmodified thyrotrophic hormone.

The maximum concentration of radioiodine in the thyroids of chicks on an iodine-poor diet occurred at 3 hours. The results obtained with one injection of the acetylated pituitary preparation prior or subsequent to the administration of radioiodine in animals on a low iodine diet are shown in Table IIIB. Injection of the acetylated pituitary preparation 3 to 18 hours prior to the ^{131}I resulted in marked elevation in the thyroidal concentration of ^{131}I. If, however, the acetylated preparation was given 3 to 15 hours after the isotope, the elevation in ^{131}I concentration in the thyroid was not as marked as with prior administration of the derivative. These data suggest that the derivative has a long latent period of action in antagonizing endogenous thyrotrophin, as reflected in the thyroidal collection of ^{131}I. Whether this same relationship would obtain in chicks on a normal diet is not yet established. There was a greater concentration when an acetylated pituitary preparation was administered prior to the radioiodine rather than afterwards (Table IIIB). This increased uptake at the time of sacrifice, 24 hours after the administration of radioiodine, might represent the stimulatory effect of unmodified thyrotrophin remaining in the acetylated pituitary preparation, or an inhibitory effect of the derivative on endogenous thyrotrophin which prevents the discharge of already localized radioiodine. Conclusive data are not available as yet to select between these two alternatives. It would appear unlikely that any significant unmodified thyrotrophin remained in the acetylated preparation inasmuch as doses of this material up to 100 times the unmodified thyrotrophin preparation failed to increase thyroid weight or ^{131}I uptake when tested over longer periods of time (Sonenberg and Money, 1957). Actually, there was a decrease in thyroid weight and thyroidal concentration of radioiodine when large doses of the acetylated pituitary preparation were injected in chicks. These latter data also make unlikely the possibility that acetylation destroyed an inhibitor already present in

the unmodified pituitary preparation thus allowing normal thyrotrophin to act in a stimulatory fashion. It would appear then that the acetylated thyrotrophin preparation because of delayed absorption, inefficient localization at the thyroid or other reasons, must be given from 3 to 18 hours prior to the administration of a tracer dose of radioiodine in order to prevent the latter from being significantly discharged from the thyroid.

Summary

The administration of an ^{35}S-labelled thyrotrophic hormone preparation to chicks has resulted in a significant concentration of ^{35}S in the thyroid. When large amounts of carrier thyrotrophin were administered with the ^{35}S-labelled preparation there was a decreased concentration of ^{35}S in the thyroid. Acetylated thyrotrophic hormone preparations were able to inhibit the action of exogenous thyrotrophin when the former was administered for 6 daily injections, as was the unmodified thyrotrophin. Other injection schedules were ineffective in inhibiting thyrotrophin administered exogenously. A single injection of acetylated thyrotrophin was able to prevent the discharge of localized radioiodine when administered from 3 to 18 hours prior to a tracer dose of ^{131}I. No other acetylated substance tested had an inhibitory effect on either exogenous or endogenous thyrotrophin.

Acknowledgements

The authors are grateful to Dr. Rulon W. Rawson for his enthusiastic support during these studies.

Misses Helen Hagopian, Alice Glattstein, Margaret Priddle, Diane Feldman, Margaret Nash and Joyce Young contributed valuable technical help in these investigations. Mrs. Renée Laqueur-Weiss was of assistance in the preparation of the manuscript.

REFERENCES

FISHER, R. A. (1941). *Statistical Methods for Research Workers. 8th* edit. Edinburgh: Oliver and Boyd.

SONENBERG, M., and MONEY, W. L. (1955). *Recent Progr. Hormone Res.*, XI, 43.

SONENBERG, M., and MONEY, W. L. (1956). *J. clin. Invest.*, 35, 736.

SONENBERG, M., and MONEY, W. L. (1957). *Endocrinology.* In Press.

DISCUSSION

Morris: Dr. Sonenberg, what was the potency of your basic TSH material you started with, approximately?

Sonenberg: Approximately 1 u./mg.

We have done experiments with thyrotrophic hormone preparations of different degrees of potency and obtained similar results. The only modification is in the reaction conditions. It sometimes was necessary to acetylate it as long as 4 hours. Sometimes we could accomplish the same results in 30 minutes.

Morris: But the correlation between the acetylation of the amino groups and the amount of inhibition would not mean a great deal in material of this potency.

Sonenberg: Not with a heterogeneous preparation like that. After acetylation there were no amino groups remaining.

Astwood: Have you tried this inhibitor in patients who have not received any tracer doses? Occasionally, patients given a tracer dose, according to Werner, will show thyroid inhibition transiently afterwards. Have you any PBI values of people not given tracer doses, but [illegible]

[illegible]

has.

Astwood: I agree; we have not seen such with tracer doses either. What sort of doses do you use?

Sonenberg: The doses range between 20 and 200 μc depending on what we do in the experiment. These were usually of the order of 200 μc.

Querido: Did you do any observations on the disappearance rate of the acetylated thyrotrophin to find out if it behaved differently?

Sonenberg: No, we did not.

Bush. Did you do any studies of the localization of the dinitrofluorobenzene derivative?

Sonenberg: No. The only localization studies we have done have been with ^{35}S-labelled preparations At present we are just checking to see if we have indeed had an inhibitor, or whether it destroyed the biological activity.

Pitt-Rivers: Do you think that the ^{35}S-label affected the protein? [illegible]

Sonenberg· Yes. We have said that many times ourselves about the limitations of using a labelled protein which is a derivative as a measure of the unlabelled proteins; so we have no illusions about that. Just circumstantially there is a lot to suggest that it does behave similarly.

Pitt-Rivers. With regard to the ^{35}S-label, do you think that it changes the molecule as little as ^{131}I-monoiodotyrosine changes plasma proteins?

For instance you can put so little iodine on a plasma protein that yo
do not alter its functional groups at all. Do you feel that you have don
the same with the ^{35}S?

Sonenberg: Not at all. We have altered it to the extent that we hav
destroyed stimulation. I think had we checked the electrophoret
mobility we should have found that we had altered it as well. A
grossly as in terms of solubility we know that we have changed it. S
it is a pretty drastic change I should venture to say.

Parkes: You have not merely re-labelled the parcel; you have change
the contents as well.

Sonenberg: But we have apparently retained something as well in so fa
as this grossly modified material still seems to localize and we canno
get anything else to localize to the same extent.

Pitt-Rivers: My feeling was that the parcel thus altered would no
necessarily go to the same place.

Bottari: Do you not have any idea about endogenous TSH secretio
when you have inhibited the thyroid response, is there an increase?

Sonenberg: The presumption is that we have inhibited the pituitar
thyrotrophin from acting on the thyroid, inasmuch as when we ad
ministered exogenous material we blocked its action. I would expec
that in these chicks where there is very little secretion during the firs
days of life there is not much change in pituitary function. It is mostl
a blocking of the action locally. We have not measured the pituitar
content of TSH.

Bottari: You don't think there is an increase in TSH?

Sonenberg: You mean after the effect on thyroid? Well, if our presen
concept of this is true, then subsequently there would be an increase in
the secretion by the pituitary. On this occasion for instance, if th
decrease in PBI was of sufficiently long standing I would expect tha
there might be an increase in TSH. We have no information abou
that.

Pitt-Rivers: Have you tried this protein in a thyrotoxic patient?

Sonenberg: We did; we just gave one injection and it actually pre-
vented the discharge of localized ^{131}I, but the patient developed fever
so we discontinued the experiment. So there is nothing conclusive
about it.

Pitt-Rivers: You didn't do an uptake at the same time?

Sonenberg: No, we did not; we just measured the discharge of hor-
monal iodine in that patient.

Ingbar: One thing that interested me was in relation to the relative
proportion of TSH which may be metabolized in the thyroid. One
would, I think, of necessity draw the conclusion, from what you say, that
that proportion of TSH which is metabolized extra-thy[illegible] ot
at all influenced by the sulphate label [illegible]

Sonenberg: I don't know [illegible]
mation [illegible]

in [illegible]

Ingbar: When it was diluted with carrier?

Sonenberg: Yes. This suggests that a smaller proportion of the labelled material was metabolized extra-thyroidally when it was diluted with carrier.

Parkes: The gonadotrophin effect was not apparently altered in these experiments?

Sonenberg: We were able to destroy the stimulatory activities after acetylation for various periods of time. In one case I showed it went down progressively after 5 hours, and if we went on for 12 hours we could destroy it completely. But at no time did we ever produce a preparation which could inhibit the gonadotrophic stimulatory response.

THE CONCENTRATION OF THYROTROPHIC HORMONE IN THE BLOOD OF THE RABBIT UNDER DIFFERENT EXPERIMENTAL CONDITIONS

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ALTHOUGH a reciprocal relationship between the activity of the thyroid and pituitary glands was suggested many years ago, and evidence is now accumulating regarding the rôle played by the hypothalamus in the regulation of thyroid function, the lack of a method, both sensitive and accurate, for determining the blood concentration of thyrotrophic hormone (TSH) has hindered detailed investigations.

In 1851 Nièpce first described hypophysial hypertrophy in cases of endemic goitre and cretinism. This observation was followed by further evidence, both morphological and experimental in type, of a pituitary-thyroid relationship. Rogowitch (1889) noted typical histological changes in the pituitary of thyroidectomized dogs and Simmonds (1914) made the striking observation that thyroid atrophy accompanies pituitary necrosis in the human. The existence of a thyrotrophic principle in pituitary tissue was first shown by Smith and Smith (1922), who found that a pituitary extract would maintain the thyroid gland of the hypophysectomized tadpole. Similar findings were made by Uhlenhuth and Schwartzbach (1926, 1927, 1928) on the salamander and larval form of the Axolotl. Crew and Wiesner (1932) made more detailed studies of the thyroid-stimulating action of pituitary extracts in hypophysectomized animals.

The discovery of a thyrotrophic principle was followed by many studies devoted to the development of an assay method for this hormone. In principle, two different techniques evolved, those depending on changes produced by an increased

blood level of thyroid hormone (indirect methods) and those based on changes produced in the thyroid gland itself (direct methods). Among the indirect methods, one of the most interesting is the "stasis tadpole method" developed by D'Angelo, Gordon and Charipper (1942). Notwithstanding its great sensitivity, it was criticized by Borell (1945) and Albert (1949) on the grounds that only direct methods should be used in a specific assay method. Among the direct methods developed during the last twenty years, the various histological techniques have been found to be reliable but often not very sensitive. The methods of Junkmann and Schoeller (1932) and Heyl and Laqueur (1935) led to the first definition of a TSH unit. Histometric methods were later found to improve the sensitivity of TSH assays, especially those in which guinea pigs (Adams and Beeman, 1942; Tala, 1945) and chickens (Rawson and Salter, 1940) were employed. The specificity of the colloid-droplet method of De Robertis (1941, 1949) and Dvoskin (1947) was questioned by Dvoskin's later work (Dvoskin, 1948). The introduction of radioactive isotopes has made possible new methods for the direct assay of TSH. In 1939, Hamilton and Soley investigated the use of ^{127}I in studies of thyroid activity. The longer half-life of ^{131}I makes it the more suitable isotope for such purposes, and measurement of the thyroidal uptake of this isotope has been employed by Ghosh, Woodbury and Sayers (1951) in the hypophysectomized rat and by Henry (1951) in the guinea pig for assaying TSH. A similar thyroidal uptake technique was used by Querido, Kassenaar and Lameyer (1953) in mice and by Overbeek and co-workers (1953) in rats after inhibition of pituitary TSH secretion by iodo-casein feeding ^{32}P uptake by the thyroid gland, as an index of TSH activity, was used by Borell and Holmgren (1946), Crooke and Matthews (1953) and by Lamberg (1953, 1955). The rate of release of ^{131}I-labelled hormone from the thyroid gland of the guinea pig (Adams and Purves, 1953, 1955) and chicken (Gilliland and Strudwick, 1953) has been used also as a measure of thyroidal activation by TSH.

The limiting factor in applying the assay methods mentioned above to the study of the TSH concentration in blood is the low sensitivity of these methods. In order to increase the sensitivity an *in vitro* method has been developed, and the account which follows is concerned with this technique (Bottari, 1956*a*, *b*). The characteristics of the method and some results obtained on the TSH concentration in the blood of rabbits submitted to various procedures which are well known to affect the rate of TSH secretion, are also described.

TSH assay by a tissue survival method

Technique

The technique in present use is as follows. Male guinea pigs weighing about 200 g. are used, since such young animals are known to have a more uniform thyroid structure than adults*. The thyroids are dissected under sterile conditions, each lobe divided into quarters sliced into small fragments. One thyroid supplies tissue for a complete assay—four tubes, two quarters per tube, i.e. two known dilutions and two different dilutions of the unknown sample. The fragments are tissue cultured for 48 hours at 37° C by means of a roller tube technique (without coagulum). The culture medium—2 ml. per tube—is composed of Gey's solution 60 per cent, horse serum 30 per cent and a solution of antibiotics (5 mg. dihydrostreptomycin and 6 mg. potassium penicillin in 10 ml. Gey's solution) 10 per cent. The pH of the medium is 7·4–7·6 as shown by the presence of the indicator phenol red in the medium. After 48 hours incubation, the TSH sample to be assayed is introduced in the incubation medium, dissolved in 0·2 ml. Gey's solution, and incubation continued for 2 hours. The medium is then replaced by an equal volume of fresh medium containing 1 μc ^{131}I per ml. and incubation continued for a further period of 1 hour. After removal of the medium the cultures are washed twice in 10·0 ml. normal saline, dried on filter paper and

* Recent experiments have shown that a single thyroid gland from a 10–12-week-old calf affords sufficient material for a large number of simultaneous cultures and avoids variation in the sensitivity to TSH.

weighed. The radioactivity of the cultures is measured by means of a well-type scintillation counter after an overnight digestion in 2N-NaOH. The results are expressed in terms of counts per minute per milligram of thyroid tissue. Two different parameters were used in plotting the log. dose response curve, either the T/M ratio (T = counts per minute per milligram of tissue; M = specific activity of an equivalent amount of medium), or the specific activity of the thyroid tissue. The first method proved to be useful in standardizing the assay technique with results pooled from different experiments, since it eliminated errors in the dilution of ^{131}I solutions. The second has been found to be reliable for the routine four-point assay procedure.

Results

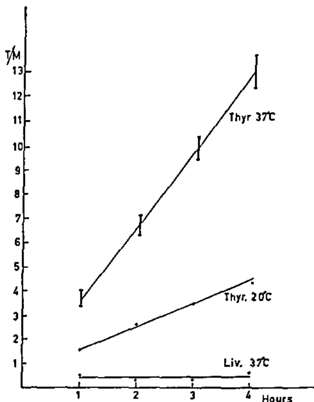
Many experiments have been undertaken to assess the reliability of the technique described above. Some of these studies will be briefly summarized.

(*a*) *Radioactive iodine uptake by thyroid tissue cultured for 48 hours.* Thyroid tissue cultured for 48 hours has still a marked ^{131}I uptake. The ability of surviving thyroid tissue to trap iodine was compared with that of other tissues (heart, liver) cultured for the same period of time in similar conditions Fig. 1 illustrates the respective uptake of ^{131}I by thyroid and liver fragments over different periods of time. While the amount trapped by the thyroid increases from 380 per cent after 1 hour to 1300 per cent after 4 hours, the amount present in the liver remains at 40 per cent during the same period. This last figure is probably due to the iodine diffusion space (figures of 22 per cent for the diaphragm, 27 per cent for the adrenals and 40 per cent for the thyroid have been obtained by Halmi, 1954).

(*b*) *The culture medium.* An absence of horse serum results in a low uptake of ^{131}I by the thyroid tissue which is in all probability due to the poor condition of the cultures. Variations in the amount of horse serum between 20 and 40 per cent seem to make little difference. An excess of penicillin is

not harmful to the cultures though an excess of streptomycin results in a change in the pH of the medium.

(c) *Concentration of radioiodine.* The concentration of radio-



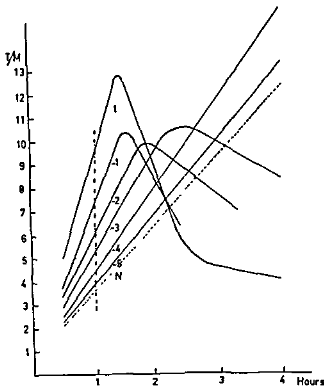
(T = counts/min./mg. tissue ; M = S.A. of an equivalent amount of medium.)

iodine may be varied between 1–12 μc/ml. without influencing the T/M ratio.

(d) *Temperature of incubation.* As may be seen from Fig. 1 the temperature of incubation markedly affects the uptake of radioiodine by the thyroid tissue. As might be expected, the Q_{10} is about 2.

(e) *The effect of TSH on* ^{131}I *uptake by thyroid tissue cultures.*

Under standard conditions of culture the uptake of radio-iodine by the thyroid tissue was found to be increased by the previous administration of TSH to the culture medium. With increasing amounts of TSH the rate of uptake increases till in all cases a maximum content of radioiodine is reached.



The time of maximum uptake occurs at different time intervals after different doses of TSH. The rate of uptake of ^{131}I and the subsequent rate of release of radioactive hormone from the gland tissue into the culture medium (as shown by butanol extraction from the medium) also vary with the TSH concentration previously applied to the tissue (Fig. 2). The log.

dose-response curve (^{131}I uptake at 1 hour period) has been found to be a straight line for doses varying from 1×10^{-5} to 1×10^{-1} i.u. TSH. A *regression curve* has been calculated and

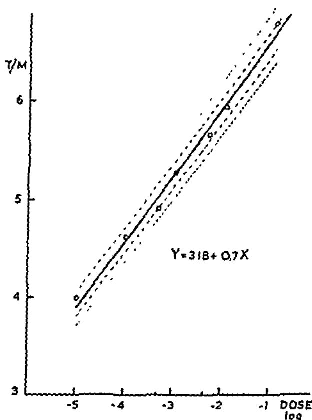


FIG. 3. Regression curve calculated from the T/M ratio after 60 minutes contact with ^{131}I after previous 2 hours incubation with increasing concentrations of TSH.

found to satisfy the equation $Y = 3{\cdot}18 + 0{\cdot}7\,X$. Analysis of variance shows that $P < 0{\cdot}001$ *for linear regression* and $P > 0{\cdot}05$ for *deviation from the regression curve*. There is then a highly significant *regression coefficient relating response to dose* (Fig. 3).

Discussion

The use of different units and of different standard preparations of TSH, by various workers, makes it difficult to compare results given in the literature. However, following the figures given by Overbeek and collaborators (1953) a rough comparison may be drawn in terms of USP standard.

Author	Figs Prov. USP Stand	Method
Bottari	0·05	^{131}I uptake—tissue survival method
De Robertis (1941, 1949)	0·1	Colloid droplets—guinea pig
D'Angelo and Gordon (1950)	0·5	Metamorphosis — tadpole
Adams and Purves (1955)	2·0	PBI release—guinea pig
Rawson and Salter (1940)	10·0	Histological—chickens
Ghosh *et al.* (1951)	10·0	^{131}I uptake—hypophysectomized rats
Borell and Holmgren (1946)	10·0	^{32}P uptake—guinea pigs
Overbeek *et al.* (1953)	10·0	^{131}I uptake—pituitary inhibited rats
Smelser (1937)	50·0	Weight of thyroids—chickens
Heyl and Laqueur (1935)	100·0	Histological—guinea pigs
Henry (1951)	100·0	^{131}I uptake—guinea pigs
Rowlands and Parkes (1934)	1000·0	Weight of thyroid—guinea pigs

In reviewing methods of TSH assay Albert (1949) has laid down various criteria which should be satisfied:

Specificity—i.e. a direct method.
Objectivity—reproducible in different laboratories.
Accuracy.

Convenience—the complete assay should be performed in a few hours, or at least in one day.
Sensitivity.

Most of these are fulfilled by the present method. Regarding convenience, once the cultures have been started the whole assay procedure only takes a few hours. Moreover, very small amounts of the sample are necessary to perform the assay, since 0·2 ml. per tube is sufficient for the determination.

The estimation of TSH concentration in the blood of normal and experimental animals

Technique

Male rabbits were used in the experiments dealing with exposure to cold, thyroxine and Pitressin injections, and female rabbits in those concerned with hypophysectomy and thyroidectomy.

Blood was collected from the marginal vein of the ear either into a heparinized syringe or heparinized centrifuge tube. After centrifugation at 3,300 r.p.m. the plasma was kept in a deep-freezer till utilization for assay.

All the TSH values are expressed in terms of international units.

Hypophysectomy. This operation was performed by the parapharyngeal route. During the approach to the pituitary gland a blood sample was obtained from the sphenoidal venous sinus in the basisphenoid bone (into which flows venous blood from the pituitary gland).

Thyroidectomy. This was performed by the classical technique, with care to avoid injury of the inferior laryngeal nerves.

Exposure to cold. Animals normally kept at a temperature of 20° C were exposed for 6 hours to an environmental temperature of 6° to 9° C. Blood samples were collected before the experiment and after 30, 60, 120, 180 and 360 minutes, exposure. One animal was transferred from a temperature of 29° to a cold room at 1° C, blood samples being collected at 0, 3, 6 and 24 hours.

Thyroxine. L-Thyroxine was injected intraperitoneally in a dose of either 50 or 100 μg.

Pitressin. This was injected intraperitoneally in a dose of 0·2 u./kg. body wt., and TSH assays made on blood samples withdrawn during the first 3 hours after injection.

Results

It has been found that the addition of known amounts of TSH dissolved in 0·2 ml. serum (horse) to the culture exerts an indistinguishable effect from the addition of the same amount of TSH in 0·2 ml. Gey's solution. In order to investigate the applicability of the method to blood of living animals a study has been made of the TSH content of rabbits' blood after various experimental procedures that are well known to affect the rate of secretion of TSH.

Hypophysectomy (Table I). Two female rabbits were

Table I

TSH CONCENTRATION IN THE BLOOD BEFORE AND AFTER HYPOPHYSECTOMY, THYROIDECTOMY AND INJECTION OF THYROXINE

TSH blood level after.	*Hours*	*Systemic blood i.u*	*Sinus blood i.u.*
Hypophysectomy	0	0 001	0 5
	24	trace	—
	0	0 007	0 04
	24	trace	—
Thyroidectomy	0	0·000	—
	24	0 002	—
	120	0·1	—
	0	0 003	—
	24	0 004	—
	120	0 1	—
Thyroxine	0	0 0004	—
	3	0 00001	—
	0	0 0007	—
	3	0 00001	—
	0	0 0005	—
	3	0 00003	—

hypophysectomized. It is of interest that high amounts of TSH were found in blood from the sphenoidal sinus as compared with systemic blood. Although the systemic sample was collected before anaesthesia, while the sphenoidal sinus sample was collected during the course of the operation, it is probable that the difference observed is significant since anaesthesia and operative trauma probably result in a decreased secretion of TSH. Twenty-four hours after hypophysectomy the content of TSH in the blood had fallen to the limit of the assay method ($< 1 \times 10^{-5}$ i.u. TSH/ml. plasma).

Thyroidectomy (Table I). Two thyroidectomized female rabbits were studied. Twenty-four hours after operation there was no change or a slight decrease in the amount of circulating TSH, but after 5 days the TSH concentration level increased to more than 1×10^{-1} i.u./ml. plasma. The slight decrease observed 24 hours after surgical removal of the thyroid may be due to the liberation of high amounts of thyroxine during the operation or to inhibition of TSH secretion by the surgical trauma.

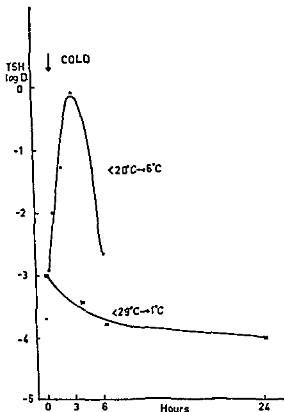
Thyroxine (Table I). The blood concentration of TSH was investigated in 5 rabbits after the intraperitoneal injection of 100 µg./kg. L-thyroxine. Blood was collected before injec*tion and 3 hours later. A significant decrease in TSH* blood level was observed.

Exposure to cold. Nine male rabbits, usually kept in an environment at 20° C, have been studied during exposure to an environmental temperature of 6–9° C. All animals showed an increased blood concentration of TSH (Fig. 4). The concentration of TSH, already increased 30 minutes after the beginning of the exposure, reached a maximum after 3 hours. By 6 hours a decrease from the maximum value was observed. One female rabbit was taken from a temperature of 29° C and placed in an environment at 1° C. This animal showed a decreased concentration of TSH in the blood, which was significant after 3 hours.

Exposure to cold after injection of thyroxine. Thyroxine, in a dose of 50 or 100 µg., was injected either just before the expo-

sure of the animal to cold, or 3 hours prior to the change in environmental temperature.

(1) The injection of thyroxine immediately before exposure



to a temperature of 6° C does not prevent the increased blood concentration of TSH. Neither the latent period of the response, nor the speed of the reaction appears to be affected, but the maximum blood concentration reached is slightly lower than normal (Fig. 5).

(2) As already stated, the injection of either 50 or 100 μg. thyroxine/kg. body wt. is followed by a significant decrease in the concentration of blood TSH. However, exposure to

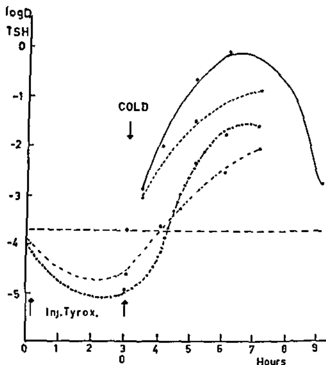


FIG. 5. TSH concentration after the exposure to a cold en-

– – – –Injection of L-thyroxine simultaneously with exposure to cold
– –·–·–Injection of L-thyroxine 3 hours before exposure to cold.

cold 3 hours later nevertheless results in an increased concentration above normal limits, although the rate of increase is very slow and there is only a tenfold increase after 3 hours instead of the 500-fold increase observed in non-treated rabbits.

Pitressin. Determinations of the TSH blood concentration

were performed on 7 rabbits after intraperitoneal injection of Pitressin at a dose level of 0·2 u./kg. body wt. Samples collected at 1, 2, and 3 hours showed a definite increase

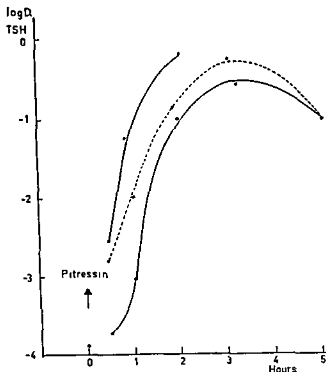


FIG 6. TSH concentration in the blood after the injection of Pitressin in a dose of 0 2 μ/kg. body weight. Dotted line—TSH concentration in the blood during exposure to a cold environment.

in circulating TSH, similar to that observed after exposure to cold. Only 1 animal out of 7 gave an equivocal response (Fig. 6).

Discussion

Few quantitative data have been published on the TSH blood concentration in animals, and the use of different

standards and units and of different assay methods makes it difficult to compare the available results.

As far as hypophysectomy is concerned, D'Angelo and Gordon (1950) did not find any TSH in the blood of hypophysectomized rats, although the normal value for this animal was found to be 0·06 Junkmann-Schoeller (J.S.) units/ml Studies based on ^{131}I uptake by Ghosh, Woodbury and Sayers (1951) show a 50 per cent decrease 24 hours after hypophysectomy.

The effect of thyroidectomy on the pituitary secretion of TSH has been inferred from indirect data. Early reports by Houssay (1932) and Hohlweg and Junkmann (1933) postulated the absence of increased TSH secretion after thyroidectomy in contradiction with the findings of Rogowitch (1889), Hofmeister (1892), Leonhardt (1897), Herring (1908) and Degener (1913) who found morphological signs of hypophysial hyperactivity after thyroidectomy. Gordon, Goldsmith and Charipper (1945) published quantitative data showing a high increase in the TSH concentration in the blood as well as in the pituitary gland 48 hours after surgical thyroidectomy. Increase in blood values was also found by Adams and Purves (1955). It is difficult to understand the decrease in blood and pituitary TSH found by Gordon, Goldsmith and Charipper (1945) after thiouracil and sulphadiazine treatment. The authors postulate an increased metabolism of TSH by the hyperplastic thyroid. However, methylthiouracil treatment was followed by an increased concentration of TSH in the blood in the experiments of Adams and Purves (1955). From the present data it seems that TSH secretion is temporarily inhibited during the first 24 hours after thyroidectomy but that after this period high concentration of TSH in the blood occurs. Brown-Grant, von Euler, Harris and Reichlin (1954) from a study of the ^{131}I-release curve in the rabbit found that injection of thyroxine was followed by an inhibition of thyroid activity, starting about 2 hours after the injection. Results obtained after thyroxine injection demonstrate a definite decrease in circulating TSH. There are no quantita-

tive data on the concentration of TSH in the blood during the first hours of exposure to cold. Brolin (1946) has shown an increase in circulating TSH after a prolonged exposure to a cold environment, and Stevens and co-workers (1955) have published figures on the TSH blood concentration after 24 hours' exposure of guinea pigs. These last authors found a twofold increase. The experiments of Brown-Grant and co-workers (1954) demonstrated that an increased thyroid activity began about 4 hours after cold exposure. Since these workers produced evidence that the latent period of action of TSH is 1–2 hours, it might be expected that a maximal increase in TSH blood concentration would be found between 2 and 3 hours after the beginning of cold exposure. The figures obtained in the present study after 3 hours' exposure to a moderate degree of cold show that this is so. The consequent decrease below the maximum figures found at 6 hours may be explained by:—

(i) a secondary inhibition due to the increase in thyroxine concentration in the blood at that time, and the stabilization of the TSH secretion rate, or
(ii) an increased rate of TSH metabolism by the activated thyroid gland.

The decrease in TSH blood concentration found after exposure to severe cold is in agreement with the findings of Brown-Grant, Harris and Reichlin (1954*a*, *b*) in the rabbit and Brown-Grant (1956) in the rat It is of interest to note that an increase in TSH blood concentration may be independent of the thyroxine blood concentration, since thyroxine injection simultaneously with cold exposure does not impair TSH secretion, and even animals injected 3 hours prior to cold exposure, in which pituitary secretion is inhibited, still respond to cold stimulus with an increase in concentration of TSH in the blood. These findings indicate that, at least in the response

definite conclusions from the observation that Pitressin injection is followed by a definite increase in TSH blood concentration. However, this fact, together with the observation that Pitressin also increases ^{131}I uptake by the thyroid (Froja and Martini, 1953; Dubreuil and Martini, 1956) provides a promising starting point for further studies.

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REFERENCES

ADAMS, A. E., and BEEMAN, E. A. (1942) *Endocrinology*, **31**, 128
ADAMS, A. E , and PURVES, H D (1953). *Proc Univ. Otago med. Sch*, **31**, 38.
ADAMS, A E , and PURVES, H. D (1955) *Endocrinology*, **57**, 17.
ALBERT, A (1949) *Ann. N.Y. Acad Sci* , **50**, 466.
BORELL, U (1945). *Acta med scand* , suppl. 161.
BORELL, U., and HOLMGREN, H. (1946). *Acta endocr., Copenhagen*, **3**, 331
BOTTARI, P. M (1956*a*). *Arch. int Physiol.*, **64**, 117
BOTTARI, P. M (1956*b*) *Arch. int. Physiol.*, **64**, 511.
BROLIN, S. E (1946) *Acta Anat.*, **2**, suppl. 3, 165.
BROWN-GRANT, K. (1956) *J. Physiol.*, **131**, 52.
BROWN-GRANT, K., VON EULER, C., HARRIS, G. W., and REICHLIN, S. (1954) *J Physiol.*, **126**, 1.
BROWN-GRANT, K , HARRIS, G. W., and REICHLIN, S. (1954*a*). *J. Physiol* , **126**, 29.
BROWN-GRANT, K., HARRIS, G W , and REICHLIN, S. (1954*b*). *J. Physiol.*, **126**, 41.
, 777.
on Colloquia
, **46**, 39.
)42). *Endo-*
DEGENER, L M. (1916)
DE ROBERTIS, E. (1941). *Amer J. Anat.*, **68**, 317.
DE ROBERTIS, E. (1949) *Ann N.Y. Acad. Sci.*, **50**, 317.
DUBREUIL, R., and MARTINI, L. (1956). *XXth Int. physiol. Congr.*, p. 257.

DVOSKIN, S. (1947). *Endocrinology*, **41**, 220.
DVOSKIN, S. (1948). *Endocrinology*, **43**, 82.
FROJA, A., and MARTINI, L. (1953) *Arch. int. Pharmacodyn.*, **93**, 167.
GILLILAND, I. C., and STRUDWICK, J. I (1953) *Clin. Sci.*, **12**, 265.
GORDON, A. S., GOLDSMITH, E. D., and CHARIPPER, H. A. (1945). *Endocrinology*, **36**, 53.
GHOSH, B. N., WOODBURY, D, and SAYERS, G. (1951). *Endocrinology*, **48**, 631.
HALMI, N. S. (1954). *Endocrinology*, **54**, 97.
HAMILTON, J. G., and SOLEY, M. H. (1939). *Amer. J. Physiol.*, **127**, 557.
HENRY, R. (1951) *Ann. pharm. franç*, **9**, 724.
HERRING, W. (1908). *Quart J. exp Physiol.*, **1**, 281.
HEYL, J. G, and LAQUEUR, E. (1935). *Arch. int. Pharmacodyn.*, **49**, 338.
HOFMEISTER, T. (1892). *Fortschr. Med.*, **4**, 122.
HOHLWEG, W., and JUNKMANN, W. (1933) *Pflugers Arch. ges Physiol*, **232**, 148.
HOUSSAY, B. A. (1932) *C. R. Soc. Biol., Paris*, **111**, 830
JUNKMANN, K., and SCHOELLER, W. (1932). *Klin Wschr.*, **11**, 1176.
LAMBERG, B. A. (1953). *Acta med. scand.*, suppl 279.
LAMBERG, B A. (1955). *Acta endocr., Copenhagen*, **18**, 405.
LEONHARDT, H. (1897). *Virchows Arch.*, **149**, 341.
NIÈPCE, B (1851) Traité du Goitre et du Cretinisme. Paris.
OVERBEEK, G A, FOKKENS, J., QUERIDO, A, DE VISSER, J., and CANNINGA, P. (1953) *Acta endocr, Copenhagen*, **14**, 285
QUERIDO, A, KASSENAAR, A. A. H., and LAMEYER, L. D. F. (1953). *Acta endocr., Copenhagen*, **12**, 335.
RAWSON, R W, and SALTER, W. T. (1940). *Endocrinology*, **27**, 155.
ROGOWITCH, N (1889). *Beitr. z. path. Anat*, **4**, 453.
ROWLANDS, I. W., and PARKES, A. S. (1934) *Biochem J.*, **28**, 1829.
SIMMONDS, M. (1914) *Deutsch. med Wschr*, **40**, 322.
SMELSER, G K. (1937) *Proc. Soc. exp. Biol, N.Y.*, **37**, 388.
SMITH, P. E, and SMITH, I P. (1922) *J. metab. Res.*, **43**, 267.
STEVENS, C. E, D'ANGELO, S. A., PASCHKIS, K. E, CANTAROW, A. S., and SUNDERMAN, F. W. (1955) *Endocrinology*, **56**, 143.
TALA, P (1945) *Acta endocr, Copenhagen*, suppl. 9, 12
UHLENHUTH, E., and SCHWARTZBACH, S. (1926). *Anat. Rec*, **34**, 119.
UHLENHUTH, E., and SCHWARTZBACH, S (1927) *Brit. J. exp. Biol.*, **5**, 1.
UHLENHUTH, E., and SCHWARTZBACH, S (1928) *Proc. Soc exp. Biol, N.Y.*, **26**, 152.

DISCUSSION

Loraine. Have you compared the sensitivity and specificity of your

Junkmann-Schoeller (J. S.) units without reference to the TSH preparation used. It is of course often difficult to compare results obtained with standards of different potency and using different units.

Roberts: There have been several reports now using assay methods depending upon thyroidal ^{131}I uptake or release which have indicated that the blood level is somewhat less than 1 mu./ml. in the rat, the rabbit and the mouse.

Bottari: For the rabbit the results I got in Brussels are about 0·5 mu./ml. plasma.

Heller: What dose of Pitressin did you use?

Bottari: 0·2 u./kg.

Heller: Have you varied the dose?

Bottari: No. I used that dose to compare the results with the same dose by Martini

Querido: I wonder whether the precision of the method allows you to distinguish between doses that differ less than with a factor 10. Your dose response runs from 10^{-5} to 10^{-1}.

Bottari: The statistical analysis of the method shows that a fourfold

Bottari: No, I have two known samples and one unknown sample diluted by 10. So I have a curve going between two known samples, and then I have the same unknown sample with and without dilution.

Diczfalusy: So it is actually a 4-point assay. Dilution is 1 to 10, both for the standard and unknown.

Bottari: Yes.

Astwood: I should like to know more about this magnificent technique. Are there any follicles left uncut? And does the tissue actually grow under these conditions?

Bottari: Under these conditions I don't have tissue growth because I use a 48-hour culture and I don't add any embryo extract to the tissues

since the time of culture is not long enough

Astwood: Most of the tissue is made up of intact follicles?

Bottari: Yes, well, there are no follicles in the inner part, but the follicles are in the outside part of the tissue.

Ingbar: Was the two hours' incubation with TSH chosen for some

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^{131}I.

Pitt-Rivers: You show no effect after thyroidectomy at 24 hours and an effect at 120 hours Have you any points in between?

Bottari: No, I just waited for the animal to recover.

Foglia: Have you any data on diabetic rats?

Bottari: No, we have just studied hypophysectomy, thyroidectomy, and cold.

Sonenberg: I don't know about the effective half-life of hormonal iodine in rabbit blood. I just wonder if there is any information about the PBI or the blood levels in the thyroidectomized rabbit at 0, 24, and 120 hours. How rapidly does the PBI decrease after thyroidectomy in rabbits? I am trying to see if I can correlate the PBI fall with the TSH increase in blood

Bottari: I have information about TSH levels but not about PBI levels

Ingbar: Do you have any data on the level of TSH after sustained exposure to cold?

Bottari: No, I stop my exposure to cold after 6 hours, and severe cold

Parkes: Do you know if you shifted their body temperature by that exposure to environmental cold?

Bottari: No, I did not take any internal temperatures.

Pitt-Rivers: Did the rabbit that was severely chilled look ill? Was it any the worse for it?

Bottari: Well, it did not look very pleased!

Pitt-Rivers: But it recovered?

Bottari: Yes.

Harris: Rabbits don't show much effect after 24 hours at 1°.

Parkes. Unless it is a very sudden change they wouldn't, because they

ill effects.

Pitt-Rivers: Except displeasure!

M. de Escobar. Do you have also any data on the changes of serum TSH in thyroidectomized animals, who have been on a constant dose of thyroxine, after exposure to cold?

Bottari: No.

Samuels: Was there any correlation between the activity of the animals and the course of TSH? Were these animals constrained, or were they able to move around?

I might mention one other method that might be suitable for blood measurements of thyrotrophin. It is the method of Adams and Purves essentially, the thyroid is labelled, suppressed with exogenous thyroid and then the blood concentration of radioactivity is measured. After an intravenous injection of thyrotrophin there is a rise in the radioactivity of the blood. This is a very sensitive method. Dr. Purves tells me that many people, in various parts of the world that he has recently visited, told him that they were unable to repeat this, and the reason he gave was that they could not handle guinea pigs in the expert way that Dr. Adams handled them. Indeed Purves says he couldn't do it himself. In Adams and Purves directions, the animals were heated, wrapped up, bled from the cut ear and prepared for injection, and then they say, "The guinea pig should be quietly dozing at this stage". Anybody else's guinea pigs are struggling and kicking!

Well, what I wanted to say was that Dr. Maxwell McKenzie from Dundee has been with us this year, and he has adapted the method to the mouse. It doesn't seem to matter whether the mouse is quiet or not —the sensitivity of the test adapted to the mouse increases so that the limit of detection is about 0 025 mu. Dr. McKenzie hasn't yet detected thyrotrophin in blood of normal human beings, but apparently it is readily detectable in myxoedema.

Querido: How steep is the dose-response curve?

Astwood. It is a steep dose-response with a limited range, which probably is an advantage.

Querido: You mean a factor of 1 to 10 for the dose?

Astwood: Not much more than that; I would guess about 1 to 20.

Parkes Is this some particular variety of mouse?

Astwood. As far as I know it is just mice.

Loraine: Do you know if a method of this type works in rats as well as mice?

Astwood: He hasn't used rats The rat is extraordinarily insensitive by other criteria.

Bottari. I suppose rats need to be hypophysectomized first.

Harris: Is the technique that you take mice, inject them with radioactive iodine, block their pituitary secretion with exogenous thyroxine, and then measure the thyroidal release of hormone using the blood radioactivity?

Astwood: Yes, that is a great deal more sensitive than trying to measure a change in the neck. A sample of blood is taken at zero time, and again at the time of maximal response two hours later; the animal can be used again next day when the blood level has again fallen to normal, and yet once more on a third day.

GENERAL DISCUSSION

Diczfalusy: Dr. Samuels asked this morning whether a low concentration of a hormone at the site of production would mean a very rapid discharge, or would mean that the production is indeed very low. Fig. 1 indicates the concentration of human chorionic gonadotrophin (HCG) per g. (wet weight) hydatidiform mole tissue (closed

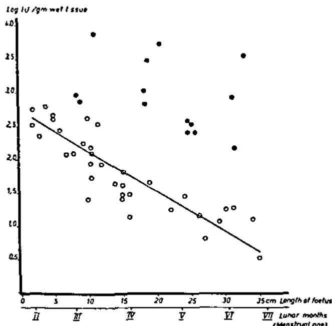


Fig 1 (Diczfalusy)

circles) and

increased hormone production, at least as far as the elaboration of HCG is concerned.

The other question was this: in the definition of an endocrine organ we often use the criterion that if an organ is involved in the production of a certain hormone one would expect that the concentration of the active substance is much higher in the venous blood leaving the organ than in the arterial blood. Thus, Dr. Borell, Dr. Fernstrom and I have conducted some experiments in cases of sus- [illegible]

taneously, and the concentration of HCG in the venous blood, the arterial blood and the urine was estimated.

The results are shown in Tables I and II.

Table I

Case No 1	*Concentration i.u./l.*	*Fiducial limits of error (P=0 95)*
Venous	438,000	(342–546)
Artery	457,000	(405–515)
Urine (24-hr.)	113,000	(88–146)

Now we repeated this in another case where there was some doubt as to whether it was a mole or a normal pregnancy, and the figures were:

Table II

Case No. 2	*Concentration i u./l*	*Fiducial limits of error (P=0 95)*
Venous	29,500	(24–36)
Artery	25,500	(22–30)
Urine (24-hr.)	18,000	(15–22)

Loraine: Were these assays conducted by the method depending on the enlargement of total accessory reproductive organs in intact immature rats?

Diczfalusy: Yes, and the values were generally around 0·10–0·12, or so.

Loraine: Was litter-mate control used?

Diczfalusy: Yes.

suppose you would not expect to see much difference.

Diczfalusy: I agree.

Astwood: The contribution would need to have been only very little.

wondered whether the destruction of the hormone is really as rapid as it was previously assumed. In the early experiments of Zondek and a number of other investigators it was claimed that if you inject human chorionic gonadotrophin you will recover something like 10 per cent.

Samuels: I have some further evidence that the HCG is not rapidly destroyed. In some experiments we have cannulated the spermatic vein of dogs and measured the output of testosterone and androstenedione in the blood flowing from the testis. We obtained low and irregular levels in the unstimulated mongrel dogs we get; the conditions under which they have been kept may determine this great irregularity in normal production. But when we made a single injection of HCG into the vein, within the first half-hour there was a significant increase in output; by the second half-hour it reached a level which was maintained for at least the next 2·5 hours. We then tried constant infusion to see whether this effect could be obtained at a lower dose of HCG if destruction was offset by administration. We found that the increase in output was delayed until we had infused between 4–8 i.u., a level that was comparable to the minimally effective single injection. Our conclusion was that we had to reach a certain amount of HCG in the circulating blood before response was obtained, and this could be achieved by the same

trophic hormone remains longer in the circulation than other types of

at least in Dr. Loraine's and in my opinion (Diczfalusy, E., and Loraine, J. A. (1955). *J. clin. Endocrin.*, 15, 424).

Loraine: When you administered HCG to pregnant subjects did you study its effect on the urinary excretion of oestrogens and pregnanediol? Was any effect observed?

Diczfalusy: We have not studied pregnanediol; we have studied oestrogens in only two cases, and I do not think that the oestrogen assays showed anything of particular interest.

Bush: I have just done a brief calculation which shows that Dr.

[illegible]

Diczfalusy: Yes, if you draw that conclusion, but you would agree the placenta of the mole tissue is, in fact, the site of the production

[illegible]

Samuels: I think Dr. Diczfalusy's conclusion that it does not apply is not correct because as Dr. Bush pointed out, the addition would be so small in relation to the total that you could not find it in collections over such short intervals. The half-life is much longer here than the half-life of a steroid, which in the case of testosterone is a matter of a very few minutes.

Pearlman: The contrast in turnover time between HCG and progesterone, both hormones arising from the placenta, is a very

[illegible]

ments are being donated to the total circulating HCG by the placenta; none the less it should be apparent that the placenta is producing HCG.

Querido: I wonder if Dr. Loraine will agree with me; I have the feeling that the Cohn fractionation is getting in fashion for other hormones than TSH, and you mentioned that there may be a fairly big loss. Our experience is that if you do this Cohn fractionation

[illegible] this [illegible], to- [illegible] be the reason for your loss.

[illegible]

The figures I quoted were from a paper by Gemzell and his collaborators in Stockholm. If I remember correctly they claimed that the total yield of the hormone using the Cohn procedure was approximately 50 per cent. I agree with Prof. Quendo that the Cohn technique is becoming more and more popular for the preparation of pituitary hormones from blood. Quite recently Dr. Janet MacArthur and her associates in Boston have used it, apparently with some success, to extract pituitary gonadotrophins from human plasma. Their assay methods depended on the enlargement of the uterus and ovary in intact female immature rats. The bulk of the activity was found in fractions I and II.

Quendo What I mean to say is, whether they are aware of the fact that you should not hunt after one specific fraction with Cohn's fractionation because this may be unsatisfactory.

Loraine: If I remember correctly I think that Dr. Gemzell found growth-promoting activity mainly in fractions IV-1 and IV-4.

Heller: I think there is an additional side to this. Has it not been shown—when the protein binding of thyroid hormones was investigated—that the binding by and hence the percentual distribution in *the various plasma fractions depends on the quantity of hormone* present?

Pitt-Rivers: Yes, but as far as natural hormone is concerned, you get the largest part in the—well actually, as Dr. Ingbar has shown—Cohn fraction IV-6, most of it, and some of it in IV-7 and IV-9.

Ingbar: I think we should distinguish, however, between situations in which the hormone is itself a protein and those in which the hormone is merely transported or bound by proteins.

Roberts: I don't agree that the recovery of circulating hormones by the Cohn fractionation procedure is necessarily anything less than 100 per cent. In fact under some circumstances more than 100 per cent of the original plasma activity may be recovered, possibly due to potentiation of hormonal activity when associated with certain plasma proteins. We have carried out work on the localization of endogenous TSH and ACTH in the various plasma protein fractions of the rat, and in both instances we have been able to recover 100 per cent of the hormonal activity present initially. Most of the activity appeared, in both cases, to be associated with the albumin

fraction although there was also some localization of TSH in the β-globulin fraction.

It should be noted, however, that the TSH assays were conducted on rat serum obtained from animals with pituitary thyrotrophin-secreting tumours. The ACTH studies were carried out on plasma protein fractions of adrenalectomized rats. Thus in neither instance were "normal" animals employed.

Heller: Did you get any difference in the binding by your albumin fraction and your β-globulin fraction with changes in the concentrations of the hormones?

Roberts: Yes, but the interpretation of such observations was com-

the endogenous.

Heller: That may have made a difference.

Proc. R. Soc. Med., 49, 209), and we even had one serum where we could extract it straight away from the paper where it was mainly located in the globulin fraction. In this serum the level was 60 times normal, and on fractionation it did not come out in the albumin fraction. That holds for endogenous human material

Roberts: There may of course be a considerable species difference as well. There seems to be an indication from other types of work

speculations as to why.

Szego: That was exogenous hormone added to the blood, wasn't it?

They have never succeeded in localizing endogenous circulating oestrogen in the human

Savard: If I recall, it was administered just prior to withdrawal of the blood.

Szego: And could have been extensively metabolized, therefore, prior to isolation from blood which was taken two hours after administration of the steroid.

Sonenberg Recently Postel has reported that there is actually an inhibitory substance using ^{131}I as a measure I think it was in pituitary and in blood as well. I wonder if those people who are using paper electrophoresis the way he did have run into this difficulty of an inhibition of ^{131}I uptake with the method used.

Roberts · I th [illegible]

results which [illegible]

encountered. [illegible]

procedure, or of the data for that matter, would always substantiate such interpretations. However, I believe that in *in vitro* assays of blood hormones in particular, potentiation or inhibition of homologous and heterologous proteins may be of considerable importance.

Morris. I think in view of what Dr. Sonenberg said earlier, in- [illegible]

Querido: Tables I and II indicate my point about the presence of

Table I (Querido)

TSH ACTIVITY IN SERUM PROTEIN FRACTIONS (COHN'S PRECIPITATION AND ELECTROPHORESIS)

Post-operative hyperthyroidism (Mrs W)

Fraction	*mg protein in 30 ml.* Albumin	$\alpha_1\alpha_2$	β	γ	$\%^{131}I$	*TSH* μg.%*
			126			
II+III	78	33	126	417	3 7±0 4	—
IV–1	54	39	12	12	3 0±0 2	—
IV–4	36	54	51	—	12 0±0 9	90–150
V	813	33	33	—	3 7±0·3	—
	981	159	222	459		
Serum equal quantity	1,101	183	231	498		

* U.S.P. standard.

TSH in different Cohn fractions. Both are cases of post-operativ hyperthyroidism. In the first one TSH is found in fraction IV-4, in the second in II + III.

Table II (*Querido*)

TSH ACTIVITY IN SERUM PROTEIN FRACTIONS (COHN'S PRECIPITATION AND ELECTROPHORESIS)

Post-operative hyperthyroidism + local myxoedema (Mr. V.)

Fraction	*mg. protein in 30 ml.* Albumin	$\alpha_1\alpha_2$	β	γ	$\%^{131}I$	*TSH* $\mu g.\%$*
II + III	84	30	147	462	30.1 ± 3.2	$\geqslant 360$
IV–1	72	69	15	9	2.1 ± 0.2	—
IV–4	24	66	51	—	3.7 ± 0.3	—
V	540	27	60	—	1.9 ± 0.2	—
	720	192	237	471		
Serum equal quantity	978	218	300	408		

* U.S.P. standard.

Pitt-Rivers. Endogenous hormone?

Querido. Yes, and you cannot predict where it is.

Szego: I should like to ask Dr. Sonenberg, and perhaps others here, why they place so much emphasis upon the quantitative localization of the hormone in its target organ, when in reality it is quite possible that qualitative association with particular reactive sites, not necessarily reflected as gross accumulation, may be the key to understanding how these substances act locally. Thus there are many examples of accumulation in such apparently non-specific loci as the liver, in which it is likely that fairly non-specific processes quite unrelated to the true mechanism of action of the hormone are going on. Dr. Sonenberg made quite a point about the retention of certain localizing properties in the modified preparations.

Sonenberg: I have said in the past that I certainly wouldn't rely on localization, alone or related to another tissue or by any other criterion, as sole criterion that a trophic hormone is related to a target gland or to a tissue. Certainly I would rely on all the other techniques which have been developed, particularly as to whether an effect has been induced, or whether any changes either biochemical or morphological have been induced. I would agree with you that there may well be changes in other tissues where localization is minimal. For instance, in some of the ways we have approached the problem using radioactivity to detect localization, knowing full well that we have destroyed the stimulatory property, we found a significant localization, as you said, in the liver, as has Williams and other people who

have tried labelling proteins. But I would not conclude that TSH acts on the liver until I could see some kind of action.

Szego: To clarify further, what I have referred to perhaps was the inconsequentiality of quantitative localization as an index of effectiveness. You use that as the prime criterion of a property of the original hormone that has been retained in the modified material. It may be quite coincidental.

Sonenberg: It is not conceivable to me that the material would not go there, yet have an effect; it is conceivable that it could have a

Szego: I merely meant that perhaps the emphasis that you were

agree.

Sonenberg: I think that we have data in support of your position with our labelled ACTH preparations, where I think, if I remember the data, that the concentration of radioactivity in the adrenal was not greater than in blood, or it may have been just slightly greater. In terms of the total amount administered, the amount which went to the adrenal was almost inconsequential, yet there was not much

property, no matter of what magnitude, may be retained, and yet the stimulatory activity of the hormone be destroyed.

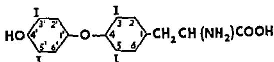
THYROID HORMONES IN THE BLOOD

ROSALIND PITT-RIVERS

National Institute for Medical Research, Mill Hill, London

The Thyroid Hormones

IT is now nearly thirty years since Harington (1926; Harington and Barger, 1927) demonstrated that the principal hormone of the thyroid, thyroxine, was β-[3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxyphenoxy)phenyl]alanine:



Harington further suggested that thyroxine was formed in the thyroid by the condensation of two molecules of diiodotyrosine, the only other iodinated amino acid then known to occur in nature.

This idea was supported by the subsequent isolation (Harington and Randall, 1931) of diiodotyrosine from the gland, and by the demonstration (Canzanelli, Harington and Randall, 1934) of the configurative relationship of thyroxine with tyrosine. Thyroxine and diiodotyrosine were both bound in peptide linkage in the protein thyroglobulin, and could only be identified after enzymic or alkaline hydrolysis.

Many years were to pass before the nature of the circulating thyroid hormone was finally established. Thyroxine itself was considered, but its long latent period of action, and a discrepancy between its activity and that of whole thyroid substance cast some doubts on its being the peripheral hormone. Some workers thought that thyroglobulin might be the circulating hormone—others favoured a thyroxine polypeptide. In 1939 Trevorrow showed that the endogenous hormone in the blood possessed thyroxine-like properties, and in 1940 Lerman

showed by immunological methods that the hormone in the blood could not be thyroglobulin. The most convincing evidence that thyroxine itself was the circulating hormone came from the work of Taurog and Chaikoff (1948). These authors found, in a number of chemical tests, that the hormone in the blood behaved in a manner identical with synthetic thyroxine.

The use of ^{131}I and chromatographic analysis opened new vistas in the thyroid field. In 1948 Fink and Fink showed that monoiodotyrosine was present in the thyroid gland, and two years later Gross and his colleagues (1950) demonstrated the existence of hitherto unknown iodinated compounds in the thyroids and sera of rats who had received doses of ^{131}I. One of these unknown compounds was shown by Gross and Pitt-Rivers (1951) to be present also in the plasma of patients treated with therapeutic doses of ^{131}I; this compound was subsequently identified as 3:5:3′-triiodothyronine (Gross and Pitt-Rivers, 1952). At the same time, Roche, Lissitzky and Michel (1952) showed that triiodothyronine was present in thyroid glands of rats following administration of radioiodide. Subsequent work has shown that triiodothyronine is present both in the thyroid and the circulation in only small amounts compared with thyroxine; it has, however, a high physiological potency, and it is now generally accepted that the thyroid has at least two active secretions: thyroxine and triiodothyronine. The other isomer of triiodothyronine, the 3:3′:5′-compound and 3:3′-diiodothyronine have also been found in small amounts in the thyroid (Roche, Michel and Wolf, 1955), but so far only the latter compound has been demonstrated in blood (Roche, Michel, Nunez and Wolf, 1955). There is at present disagreement about the physiological activity of 3:3′-diiodothyronine; it is therefore premature to include it among the thyroid hormones. In this communication, only thyroxine and 3:5:3′-triiodothyronine will be considered.

Monoiodotyrosine and diiodotyrosine do not leave the thyroid gland under normal conditions though they are found in the blood in certain pathological states; however, both

compounds are physiologically inert, and their metabolic fate is irrelevant to the present discussion.

Thyroglobulin itself is only found in the blood after the thyroid gland has undergone trau[illegible]
Peterm[illegible]
giv[illegible]
the[illegible]
out[illegible]
[illegible]

It is likely that thyroglobulin is present in the serum of patients suffering from Hashimoto's thyroiditis at some period in the course of the disease. Owen and McConahey (1956) have found a thyroglobulin-like protein in the sera of these patients after small doses of ^{131}I. The protein was acid-soluble and not extractable with butanol, and it was considered likely to be thyroglobulin, by analogy with the findings of Robbins, Petermann and Rall (1954*a*, *b*). Doniach and her colleagues (Roitt *et al.*, 1956) have recently demonstrated the presence of antibodies to human thyroglobulin in the sera of patients with Hashimoto's disease; positive precipitin reactions were obtained by adding the sera to saline extracts of human thyroid or purified thyroglobulin. These authors consider that the destruction of the thyroid, typical of this disease, is due to interaction of the auto-antibodies with the thyroglobulin in the gland. The formation of these antibodies is presumably due to thyroglobulin leaking out of the gland in earlier stages of the disease.

Nature of the Thyroxine-Binding Protein in Blood

It has been known for some time that hormonal iodine in the blood is loosely bound to protein; it cannot be separated by dialysis and is co-precipitated by protein precipitants. Early attempts to discover the nature of the thyroxine-binding protein (TBP) by precipitation (Salter, 1949) and in the electrophoresis apparatus (Silver and Reiner, 1950) showed a distribution of iodine between albumin and the α- and β-globulins. More recently, paper electrophoresis has been used

to investigate this problem. Gordon and colleagues (1952) examined by this method the plasmas of patients who had been treated with therapeutic doses of ^{131}I. They found that the endogenous hormone was transported at pH 8·6 by a protein with a mobility similar to that of α_1-globulin. Synthetic labelled thyroxine added to normal plasma moved to the same position for the most part, though a small amount of radioactivity was present in the albumin fraction. Later work on these lines (Robbins and Rall, 1952; Deiss, Albright and Larson, 1952; Horst and Rösler, 1953) has shown that TBP migrates at pH 8·6 to a position between α_1- and α_2-globulins. A similar mobility for TBP is also found at pH 7·6 and 6; at pH 4·5 (Robbins, Petermann and Rall, 1955) it behaves electrophoretically like the α_2- glycoproteins of Schmid (1953*b*) or the M-2 glycoproteins (mucoproteins) of Mehl, Golden and Winzler (1949). The sedimentation constant of TBP labelled with ^{131}I-thyroxine is 3 5 Svedbergs (Petermann, Robbins and Hamilton, 1954).

The nature of TBP is not yet known; Robbins, Petermann and Rall (1955) have suggested that it might be a mucoprotein, because of its electrophoretic mobility at pH 4·5 and because of the observations of Mustacchi, Petermann and Rall (1954) that human serum mucoproteins are high in thyrotoxicosis and low in myxoedema. However, no difference in thyroxine-binding in sera of euthyroid and hyperthyroid patients has been found, and Ingbar (personal communication) has observed that human serum mucoproteins prepared by the method of Mehl, Golden and Winzler (1949) possess no thyroxine-binding properties.

Schmid (1953*a*) has found that his fraction VI of human plasma, a mixture of α-globulins, possesses the highest iodine–nitrogen ratio, as measured by chemical analysis; but Freinkel, Dowling and Ingbar (1955) have not confirmed this finding using ^{131}I-labelled hormone. They find the greatest enrichment of TBP in the Cohn fraction IV–6, method 6 (Surgenor *et al* , 1949) which represents a different group of proteins.

TBP has a limited capacity to bind thyroxine. This has

been shown in sera from euthyroid, hyperthyroid and hypothyroid subjects (Horst, 1954; Albright, Larson and Deiss, 1955). Freinkel, Dowling and Ingbar (1955) have found that increasing amounts of added thyroxine up to 194 µg./100 ml. displaced the endogenous ^{131}I-labelled hormone progressively from TBP to albumin. Robbins and Rall (1955*a*) have shown that protein-bound thyroxine rises in a linear manner with increasing total added thyroxine until the latter reaches a concentration of about 0·1 µmoles/l. (about 8 µg. %). Further additions of thyroxine showed a deflection of the curve, which the authors interpret as a saturation of TBP and a consequent binding by other proteins; they estimate that this saturation occurs when the thyroxine concentration has reached 2–3 times the normal level.

Triiodothyronine is also protein-bound in the blood though the attachment is weaker; Deiss, Albright and Larson (1953) showed that thyroxine could displace triiodothyronine from TBP *in vitro*, though the reverse is also true (Robbins and Rall, 1955*b*) when the concentration of triiodothyronine is raised to high levels. Dingledine, Pitt-Rivers and Stanbury (1955) confirmed the protein-binding of triiodothyronine by plasma *in vitro*; the major carrier was TBP, but triiodothyronine was also bound to albumin and other globulin fractions, even when the concentration was as low as 1·2 µg./100 ml.

The Thyroid Hormone in Pregnancy

The activity of the thyroid gland is increased during pregnancy; the gland itself is often enlarged and its ^{131}I uptake (Pochin, 1952; Ferraris and Scorta, 1955) is increased. This increase falls soon after delivery, sometimes to somewhat below normal values. Man and her colleagues (Heinemann, Johnson and Man, 1948) have shown that the protein-bound iodine (PBI) is raised in pregnancy; the rise occurs within the first month after conception, and thereafter does not alter. In sera from 29 normal pregnancies, these workers found PBI values ranging from 6·2–11·2 µg./100 ml. compared with the normal range of 4·0–8·0 µg./100 ml.

The raised PBI of pregnancy is not associated with an increased metabolic rate, at least during the first half of pregnancy: Sandiford and Wheeler (1924) found that the BMRs of pregnant women remained constant until the 7–8th month, when a slight rise occurred. After delivery there was a temporary fall in BMR to below normal levels.

The absence of calorigenic action on the mother of the increased PBI is perhaps rather surprising, and might lead to the question: is the increase in PBI an increase in thyroxine, or may it represent some non-calorigenic substance? This question has been answered; Danowski and co-workers (1950) have shown that the increased PBI in pregnancy is thyroxine, according to the methods of identification of Taurog and Chaikoff (1948). Further, Ferraris and Scorta (1955) have found increased ^{131}I-labelled thyroxine in the plasma of pregnant women after administration of ^{131}I, using chromatographic methods of identification. It seems therefore that in pregnancy the mother does not utilize the thyroid hormone in the normal way, or that she is in some way protected against it. Increased thyroid hormone appears to be essential for healthy pregnancy, and Heinemann, Johnson and Man (1948) found that in patients who were aborting or threatening abortion, PBI levels of only 2·8–5·8 μg./100 ml. were found.

Another increased function in plasma of pregnancy is its thyroxine-binding capacity. Dowling, Freinkel and Ingbar (1956*a*) have found that increasing additions of thyroxine *in vitro* to plasma labelled with ^{131}I-thyroxine revealed a greatly enhanced binding capacity of TBP; this was especially noticeable at the higher thyroxine concentrations which reached 280 μg./100 ml. The raised thyroxine-binding of plasma of pregnancy persists well into the puerperium. It is also noteworthy that these authors failed to observe any enhanced TBP activity in the plasmas of women with a diagnosis of abortion.

Dowling, Freinkel and Ingbar (1956*a*) have studied the possibility that the raised thyroxine-binding power of serum in pregnancy might result from some compensation for the

known (Longsworth, Curtis and Pembroke, 1945) lowering of serum albumin at this time. They added mercaptalbumin to pregnancy serum *in vitro* in amounts sufficient to restore the albumin to normal levels, but found that TBP activity was not affected. It appears that TBP activity in pregnancy is stimulated by some other factor.

An interesting suggestion has been made by Dowling, Freinkel and Ingbar (1956*b*) of a possible correlation between raised PBI, raised thyroxine-binding capacity and raised oestrogen levels in pregnant women's plasma. It has already been shown (Engstrom, Markardt and Liebman, 1952) that administration of 20–100 mg. diethylstilboestrol daily to normal men and women induced a rise in the PBI; after 3–4 weeks oestrogen administration the PBI values levelled off, and on stopping treatment they returned to normal values. Dowling, Freinkel and Ingbar (1956*b*) have investigated the effect of oestrogen on thyroxine-binding of serum—administration of diethylstilboestrol to euthyroid and hypothyroid subjects induced a rise in thyroxine-binding comparable to that found in pregnancy. This was also found in a case of panhypopituitarism, but was absent when studied in a thyrotoxic patient. Withdrawal of the oestrogen was followed by a return of thyroxine-binding of the plasma to normal values.

In the new-born infant Man and co-workers (1952) have found that PBI levels are raised. Dowling, Freinkel and Ingbar (1956*a*) have found that thyroxine-binding in the blood of newborn infants is also raised. These facts might indicate a transfer of thyroid hormone through the placenta. At present the evidence for placental transfer is meagre and somewhat contradictory, though Peterson and Young (1952) have produced satisfactory evidence for it in the guinea pig. Grumbach and Werner (1956) have done some short term experiments on the transfer of ^{131}I-labelled thyroxine from mother to foetus, when it was administered at from 10 minutes to 169 hours before delivery. Some serum-precipitable iodine was found in the foetal blood, even after the shorter time intervals between administration of thyroxine and delivery. The authors con-

clude that there is only a slow transfer of thyroid hormone across the placenta, but their experiments do not preclude the possibility that this transfer may indeed be taking place throughout pregnancy.

REFERENCES

ALBRIGHT, E. C., LARSON, F. C., and DEISS, W P (1955). *J. clin. Invest.*, **34**, 44.
CANZANELLI, A., HARINGTON, C. R., and RANDALL, S S (1934) *Biochem. J*, **28**, 68.
DANOWSKI, T S, GOW, R. C., MATEER, F. M, EVERHART, W. C, JOHNSTON, S. Y., and GREENMAN, J. H. (1950). *Proc. Soc exp. Biol., N.Y.*, **74**, 323
DEISS, W P., ALBRIGHT, E. C., and LARSON, F. C. (1952). *J. clin. Invest.*, **31**, 1000.
DEISS, W. P., ALBRIGHT, E. C, and LARSON, F. C (1953). *Proc. Soc. exp. Biol., N.Y.*, **84**, 513
DINGLEDINE, W S PITT-RIVERS, R., and STANBURY, J. B (1955) *J. clin. Endocrin Metab*, **15**, 724
DOWLING, J. T, FREINKEL, N., and INGBAR, S H (1956*a*) *J. clin. Invest.*, **35**, 1263
DOWLING, J T., FREINKEL, N., and INGBAR, S. H. (1956*b*). *J. clin. Endocrin. Metab*, **16**, 1491.
ENGSTROM, W W, MARKARDT, B., and LIEBMAN, A. (1952). *Proc. Soc. exp. Biol., N.Y.*, **81**, 582.
FERRARIS, G. M, and SCORTA, A. (1955). *Minerva ginec.*, **7**, 308
FINK, K, and FINK, R M. (1948) *Science*, **108**, 358.
FREINKEL, N, DOWLING, J T., and INGBAR, S. H. (1955) *J clin. Invest.*, **34**, 1698
GORDON, A H, GROSS, J., O'CONNOR, D, and PITT-RIVERS, R. (1952). *Nature, Lond*, **169**, 19.
GROSS, J, LEBLOND, C. P, FRANKLIN, A E, and QUASTEL, J H (1950). *Science*, **111**, 605.
GROSS, J, and PITT-RIVERS, R (1951). *Lancet*, **2**, 766
GROSS, J, and PITT-RIVERS, R. (1952). *Lancet*, **1**, 439
GRUMBACH, M M, and WERNER, S. C. (1956) *J clin. Endocrin. Metab.*, **16**, 1392
HARINGTON, C R. (1926) *Biochem J*, **20**, 300.
HARINGTON, C R., and BARGER, G. (1927) *Biochem. J*, **21**, 169.
HARINGTON, C R., and RANDALL, S. S (1931) *Biochem. J*, **25**, 1032
HEINEMANN, M, JOHNSON, C. E., and MAN, E B (1948). *J clin. Invest.*, **27**, 91.
HORST, W. (1954) *Strahlentherapie*, **94**, 169
HORST, W., and ROSLER, H. (1953) *Klin. Wschr.*, **31**, 13.
LERMAN, J. (1940). *J clin. Invest.*, **19**, 555.
LONGSWORTH, L G., CURTIS, R. M, and PEMBROKE, R. H. Jr. (1945). *J clin. Invest*, **24**, 46

MAN, E. B., PICKERING, D. E., WALKER, J., and COOKE, R. E. (1952). *Pediatrics*, **9**, 32

MEHL, J. W., GOLDEN, F., and WINZLER, R. J. (1949). *Proc. Soc. exp. Biol.*, **72**, 110.

MUSTACCHI, P., PETERMANN, M. L., and RALL, J. E. (1954) *J. clin. Endocrin Metab.*, **14**, 729.

OWEN, C. A. Jr., and MCCONAHEY, W. M. (1956). *J. clin. Endocrin. Metab.*, **16**, 1570.

PETERMANN, M. L., ROBBINS, J., and HAMILTON, M. G. (1954). *J. biol. Chem.*, **208**, 369.

PETERSON, R. R., and YOUNG, W. C. (1952). *Endocrinology*, **50**, 218.

POCHIN, E. E. (1952). *Clin. Sci.*, **11**, 441.

ROBBINS, J., PETERMANN, M. L., and RALL, J. E. (1954*a*). *J. biol. Chem.*, **208**, 377.

ROBBINS, J., PETERMANN, M. L., and RALL, J. E. (1954*b*). *J. biol. Chem.*, **208**, 387.

ROBBINS, J., PETERMANN, M. L., and RALL, J. E. (1955). *J. biol. Chem*, **212**, 403.

ROBBINS, J., and RALL, J. E. (1952) *Proc. Soc. exp. Biol., N.Y.*, **81**, 530.

ROBBINS, J., and RALL, J. E. (1955*a*). *J. clin. Invest.*, **34**, 1324.

ROBBINS, J., and RALL, J. E. (1955*b*). *J. clin. Invest.*, **34**, 1331.

ROCHE, J, LISSITZKY, S, and MICHEL, R. (1952). *C. R. Acad. Sci., Paris*, **234**, 1228.

ROCHE, J., MICHEL, R., NUNEZ., J., and WOLF, W. (1955). *C. R. Soc. Biol., Paris*, **149**, 884.

ROCHE, J., MICHEL, R., and WOLF, W. (1955). *C. R. Soc. Biol., Paris*, **149**, 1604.

ROITT, I. M., DONIACH, D., CAMPBELL, P. N., and VAUGHAN HUDSON, R (1956). *Lancet*, **2**, 820.

SALTER, W. T. (1949). *Ann N.Y. Acad. Sci.*, **50**, 358.

SANDIFORD, I., and WHEELER, T. (1924). *J. biol. Chem.*, **62**, 329.

SCHMID. K. (1953*a*). *J. Amer. chem. Soc.*, **75**, 60.

SCHMID, K. (1953*b*). *J. Amer. chem. Soc.*, **75**, 2532.

SILVER, S, and REINER, M (1950). *Bull. N.Y. Acad. Med*, **26**, 277

[illegible] L., GORDON, R. S. Jr., [illegible] *Soc.*, **71**, 1223.

[illegible] *Chem.*, **176**, 639.

[illegible]

DISCUSSION

Sonenberg: Dr. Pitt-Rivers raised the question about this iodine fraction which is bound to γ-globulin; Dr. Brener in our laboratory is a [illegible] concerned now whether this may be an artifact or not, and it [illegible] teins. [illegible] with [illegible] e bar- [illegible] me it

may be bound to γ-globulin instead of to albumin, as some of the curves here demonstrate.

Pitt-Rivers: It just seems unnatural saturation of thyroxine-binding protein and albumin?

Sonenberg: Yes.

Ingbar: It would be hard to account for that on the basis of barbiturates as most of the electrophoresis is done in veronal buffer.

Sonenberg: True; I am not sure that it was barbiturates but perhaps some other drugs patients had received beforehand

Astwood: Is this radioactivity bound to γ-globulin seen only when there is material that does not move on a chromatogram? Would not a thyroglobulin-like substance migrate with γ-globulin upon electrophoresis?

sera of these two patients I didn't get radioactivity at the origin; it was not butanol-extractable.

Sonenberg. In observations to which I referred the thyroxine has been added *in vitro* to the serum of cancer patients, and it seems that on a

oestrogens observed when oestriol or oestradiol were used instead of stilboestrol? Because stilboestrol is not the circulating hormone.

Pitt-Rivers: No. It is a synthetic compound.

Ingbar: Those observations are being made.

Heller. Have you any other observations on patients with low plasma protein levels or patients with hypoalbuminaemia, for instance nephrotic patients?

Pitt-Rivers. The only example I can remember at the moment has got nothing to do with thyroxine-binding protein (TBP) but it was some work of Rasmussen, where he recently demonstrated mono- and di-iodotyrosine in the urine of nephrotic patients, but I cannot call to

associated with a marked hypoalbuminaemia, excess amounts of thyroxine are frequently bound, not in the TBP spot, but shifted definitely backwards to the α_2, and it is my impression that this is what Recant was observing. Now Robbins has also studied this and has definitely concluded that there is not augmented binding to TBP in process but rather diminished binding, if I can recall correctly. That is the conclusion we would make on the nephrotic patients we have studied In addition we have been able to show a distinct amount of TBP in the urine. If you concentrate the urinary proteins you end up with a mixture of proteins which are at least as potent in binding thyroxine in the TBP zone as is the patient's own serum.

The only other situation that would fit the criteria you mention, Prof. Heller, is the patient that we have studied with a hydatidiform mole who

[illegible]

despite the addition of, as I recall, 700 or 800 µg per cent thyroxine, and despite the addition of albumin.

Pitt-Rivers: That would be the patient to have for the isolation of TBP.

Prunty: Is there any evidence that the albumin fraction in the urine of these nephrotics contained much bound hormone?

Ingbar: I don't think there is any evidence concerning the distribution of the hormone between urinary proteins.

Astwood. Has there been any evidence of an abnormal binding in hyperthyroidism? Has that been studied?

Ingbar: Yes, by a number of people. The problem that arises is that

[illegible]

might add

[illegible]

therapy. [illegible] that Dr. Ingbar has

[illegible]

the other [illegible]

thyroid to the tissue.

Ingbar: That possibility has been investigated by these relatively crude techniques, and one cannot detect any abnormality in the thyroxine-binding protein in serum of either treated patients or their relatives However, it is entirely conceivable that there is an abnor-

produced, was it?

binding protein is only one aspect of an apparent general resistance to administered hormone

Pitt-Rivers And not a peculiarity of the transporting protein?

Ingbar. I think not

Migeon. As mentioned by Dr Pitt-Rivers, the thyroxine-binding capacity of plasma protein is increased during pregnancy. If an athyreotic female patient becomes pregnant and if this patient is maintained on a constant dose of thyroid hormone one would probably expect her protein-bound iodine to go up, have such observations been reported?

And another point: clinicians who have an athyreotic patient who becomes pregnant, will usually increase the dose of thyroid hormone

patients successfully through pregnancy, and what tests to go by in order to adjust the treatment of the patient at the optimal level

the PBI. The real paradox here is that the increase in PBI in the oestrogen-treated patients is not associated with any increase in the uptake of iodine. The question which arises is where this extra circulating hormone is coming from The turnover studies are being done and we don't know as yet what effect oestrogens have on the peripheral turnover of thyroxine. However, one possibility arises in relation to the free thyroxine within

We have been able to show racteristics of a thyroxine- it combines thyroxine in a loose butanol-dissociable link.

This raises the question as to whether there is not an equilibrium across the thyroid gland itself, with competition for thyroxine between glandular thyroglobulin and circulating TBP, such that alterations in

That is purely speculative.

usual

indi- viduals?

study.

tend to men?

Pitt-Rivers: Yes.

Bush: There are quite a number of reactions of the liver with quite

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men and women.

IODINE IN BLOOD

E. B. Astwood, Carl E. Cassidy, M. S. Raben,
and Sara M. Astwood

Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts University School of Medicine, Boston

Measurement of the small quantities of iodine in the blood has long been a difficult procedure and until recently reliable values were available only from a few special laboratories using one or another exacting technique. Most values obtained prior to 1940 were, according to current estimates, either too low or much too high. The great difficulty of digesting the large amount of organic matter without loss of iodine, contamination in reagents, and the stringent requirements of an accurate titration of the micro amounts of iodine contributed to the trouble. With the introduction of the catalytic method of determination by Sandell and Kolthoff in 1937, an adequately sensitive quantitative method became available to replace titration. Chaney (1940) incorporated this into his much-used method, as did others in the several minor modifications thereof. But there still remained the problem of digestion. The cumbersome wet-ashing methods required distillation of the iodine from the large quantity of digestants, and losses were difficult to avoid. Dry ashing in alkali, though known to be associated with losses (McClendon, 1939), provided a practicable method (Barker, Humphrey and Soley, 1951) accurate enough for routine hospital laboratories. The use of chloric acid for digestion, as in the method of Zak and co-workers (1952), obviated distillation and the losses attending alkali fusion.

Analytical Method

Samples of 0·5 ml. serum and of the once-washed 5 per cent trichloroacetic acid precipitates of serum were digested with

6 ml. chloric acid on a sand bath at 140° in 40 ml. round-bottom, open-mouthed centrifuge tubes for 2 to 2·5 hours. Further chloric acid was added dropwise at intervals during the last hour of digestion to maintain the chromate catalyst in the pink, oxidized state. The final 0·5 ml. residue was diluted with 10 ml. water and the arsenious acid and ceric sulphate reagents similar to those specified by Lein and Schwartz (1951) applied to the colorimetric determination. It was found convenient for one technician in one day to carry out determinations of total and protein-bound iodine on 16 sera simultaneously, 6 tubes containing standard iodate and 2 blank tubes being included with each lot.

Range of Values

An analysis was made of the distribution of values for the protein-bound iodine including all determinations made

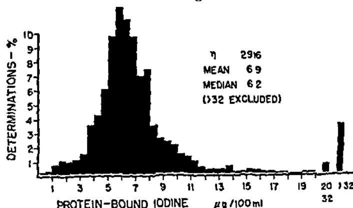


FIG. 1. Distribution of protein-bound iodine values, grouped in class intervals of 0·5 µg./100 ml. expressed as percentages of the total number of determinations carried out over a period of 19 months.

during a recent period of 19 months. The 2916 values were grouped in intervals of 0·5 µg./100 ml. and the number in each interval expressed as a percentage of the total (Fig. 1). The range extended from 0 to greater than 32 µg./100 ml. with a predominance of the values clustered about the modal value of 5·8. However, no segregation into different populations

was evident and there was not a great departure from a normal distribution.

For comparison a similar study was made of a larger series, made available to us by the Boston Medical Laboratories (Fig. 2). Again there was no division into separate populations and a normal curve of distribution was even more closely approximated. The slightly greater mean and median and the larger proportion of high values in the Hospital series is

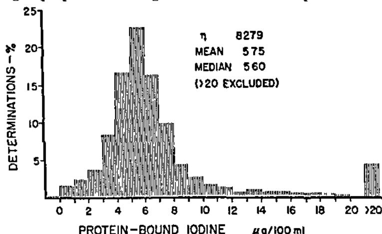


FIG. 2 Distribution of protein-bound iodine assays on samples of serum submitted to a commercial laboratory Data provided by Messrs. J and N. Benotti, Boston Medical Laboratories

probably explained by the number of cases of hyperthyroidism included in this population. There were 3 5 per cent of values greater than 32 µg./100 ml. in the former series and 4·2 per cent greater than 20 µg /100 ml. in the latter. The proportions above 20 µg./100 ml. in the two series were almost identical.

The differences between the total iodine and the protein-bound iodine corresponding to the series of Fig. 1 showed the distribution of Fig. 3. In nearly one-third of the determinations, non-protein-bound iodine was not detectable and in 87 per cent it was less than 2 µg. per cent. Slightly more than 4 per cent *showed values greater than* 4 µg. *per cent and among* these there were many cases that could clearly be attributed

to excessive iodine ingestion. Indeed, it was often possible to incriminate exogenous iodine whenever the non-protein-bound iodine exceeded 2 µg./100 ml.

The correlation coefficient between the values for protein-bound iodine and unbound iodine was calculated by the standard method and found to be + 0·03. This was taken to indicate that an insignificant proportion of protein-bound

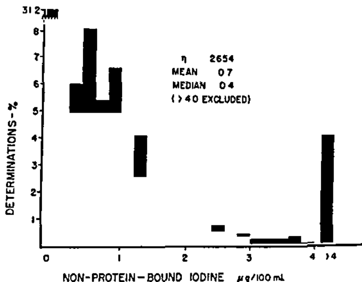


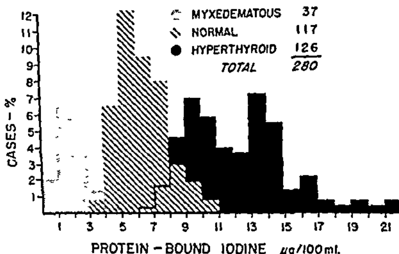
FIG. 3. Distribution of values for unbound iodine corresponding to the bound iodine determinations shown in Fig. 1.

iodine found its way into the unbound fraction, and it was inferred that the quantity of unbound iodine bore no relation to the quantity of bound iodine of presumed thyroid origin, and thus that it was not a measure of functional activity of the thyroid gland.

Hyperthyroidism and Myxoedema

Evaluation of the usefulness of these determinations in assessing the status of thyroid function was difficult because, during the accumulation of the data, the values for the protein-bound iodine were taken into account in arriving at a diag-

nosis. To avoid this potent source of error, a large number of case records were reviewed and only those cases retained wherein a definite diagnosis could be made on the basis of other criteria. Clear-cut cases of myxoedema and of hyperthyroidism were included as were individuals with no suspicion of thyroid disease. In the important group, doubtful cases, criteria for classification included studies of radioiodine uptake



by the thyroid, response of uptake to the administration of thyroid in a dose of 3 grains daily for three weeks, the response of uptake to a single injection of thyrotrophin, and response to therapy or the course of the disorder without treatment.

Selected in this way there remained 37 cases of myxoedema and 126 of hyperthyroidism while 117 had no thyroid disorder. The protein-bound iodine values were grouped in Fig. 4 in classes of 1·0 µg./100 ml. and expressed as percentages of the total of 280 patients. In the 117 individuals judged to have no thyroid disorder the protein-bound iodine fell between the

limits of 4·0 to 8·0 in 100; it was below 4 in two and above 8 in fifteen. In only one individual with myxoedema was the value above 4, and thus discrimination was good if the arbitrary value of 4 μg./100 ml. were taken as the lower limit of normal. Separation of cases of hyperthyroidism from the normal on the basis of the protein-bound iodine values was not as reliable. Inspection of Fig. 4 suggested that 8 μg./100 ml. (a commonly accepted value) would provide the most favourable differentiation, but with 18 per cent of the normal individuals falling above and 3 per cent of hyperthyroid patients below, it was apparent that the division was not sharp. A value somewhat in excess of 8·0 μg./100 ml., perhaps 8·5, might provide a better estimate of the upper limit of the normal range for diagnostic purposes.

Simple Goitre

The cases classified as simple or non-toxic goitre were those with no clinical manifestations of hyperthyroidism. In the one-third of the cases having elevated radioiodine uptakes, suppression by medication with 3 grains of thyroid daily was

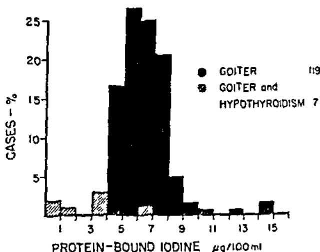


FIG. 5. Concentrations of protein-bound iodine in simple goitre and in goitre in association with hypothyroidism.

measured. Three or four instances of a single nodule, not suppressed by thyroid, were not included; the remainder showed suppression to below normal values in all instances. The distribution of bound iodine in the 126 cases (Fig. 5) showed that 90 per cent fell within the range of 4–8 µg./100 ml. in those judged clinically to be euthyroid, 10 per cent were above 8 µg./100 ml. and three patients had very high values for which there was no explanation. Of the seven patients with goitre associated with hypothyroidism, six gave values below 4 µg./100 ml. Most of the patients with goitre received thyroid medication for prolonged periods to determine whether regression could be achieved. While receiving the usual dose of 3 grains daily the protein-bound iodine usually fell within the normal range.

Thyroiditis

The clinical diagnosis of subacute thyroiditis embraced most diverse clinical pictures. Severity varied from mere tenderness of a part of the thyroid to the severe condition associated with fever. Duration and degree of involvement of the thyroid also varied. Consequently, it is not surprising that no consistent pattern of the protein-bound iodine or radioiodine uptake was observed (Table I).

Table I

PROTEIN-BOUND AND TOTAL IODINE IN SUBACUTE THYROIDITIS

These values for the 24-hour uptake of radioiodine were abnormal in 6 of the 9 cases

	Duration	PBI µg %	TI µg.%	^{131}I *uptake* %
Severe	3 weeks	17·0	18 5	1
Mild	2 weeks	11·0	11 8	0
Mild	3 weeks	9·0	—	33
Mild	1 month	6·7	7·8	0
Mild	6 months	6 4	—	43
Mild	10 months	6·7	6·7	15
Atypical	2 months	8 5	8 7	30
Atypical	3 months	1·6	1·9	11
Atypical	9 months	6·1	7 1	0

Hypopituitarism

The patients suffering from hypopituitarism also formed a non-homogeneous group. Some exhibited the full picture of Sheehan's syndrome while others with tumours, and especially those with suprasellar tumours, gave evidence of incomplete pituitary failure with deficiency of gonadotrophic and growth activities predominating. The values listed in Table II

Table II

IODINE VALUES AND RADIOIODINE STUDIES IN 6 CASES OF HYPOPITUITARISM

	PBI µg.%	*TI* µg %	^{131}I *uptake* %	*After thyrotrophin* %
Pituitary dwarf	2 5	—	20	—
Pituitary dwarf	3 2	4 0	14	52
Sheehan's syndrome	3 0	3 0	4	13
Sheehan's syndrome	1·6	1 8	3	17
Chromophobe adenoma	5·0	5 0	19	40
Probable chromophobe adenoma	3 4	3 4	47	—

suggested that the protein-bound iodine may not be quite *as* low as in myxoedema, a finding in keeping with the appreciable uptake of radioiodine by the thyroid in most cases of hypopituitarism.

Some Causes of Spurious Results

Sandell and Kolthoff (1937) showed that the catalytic effect of iodine in reduction of ceric ion by arsenite was poisoned by mercury and by other ions, including fluoride, cyanide and silver. Sandell and Kolthoff also showed that the *only substance causing catalysis comparable in rate to that* caused by iodine was osmium. Catalysis by chloride and platinum was very weak, and that by bromide also very weak unless a manganous salt was also present. *In practice only mercury* remaining in the blood 24 to 48 hours after the administration of a mercurial diuretic has been encountered *as* an interfering

ion. Sometimes contamination of the syringe by mercury has been suspected.

A very troublesome cause of error in the use of iodine in blood as a test is iodine itself. Iodine, though easily excluded from the analytical laboratory, is not easily kept away from patients. The most troublesome source of excessive iodine is radiographic contrast media, but iodides are also given for asthma and for various chronic diseases, including atherosclerosis and arthritis, and iodine is often incorporated into vitamin mixtures, cough medicines, antiseptics, parasiticides, toothpastes, and sun-tan lotions.

The substances used for displaying the urinary tract leave the body quickly and have not been found to cause erroneously high values for more than a few days. The compounds used for radiograms of the gallbladder and bile ducts are given in doses of several grams of iodine, and while fairly rapidly excreted—half-time estimated to be 12 hours for iodoalphionic acid (Slingerland, 1957)—protein-bound iodine may remain high for three months and occasionally longer. Iodized oils used for bronchograms and myelograms are very slowly absorbed and may cause false values for several years.

In most instances, the raised concentration of bound iodine when caused by excess intake of iodine was found associated with an increase in unbound iodine. The latter finding proved to be most helpful. The increased protein-bound iodine appearing on chronic administration of iodine (Danowski, Johnston and Greenman, 1950) was encountered in patients treated with cough medicines and asthma remedies containing iodide, and in patients with hyperthyroidism taking iodine. The length of treatment needed to cause the development of this form of bound iodine seemed quite variable, and the persistence of the substance in the serum after stopping the medication was more variable still. The quantities of bound iodine were not large and seldom exceeded 20 µg./100 ml., and when iodine ingestion was recent, the cause of the high value was usually revealed by the high value of unbound iodine.

An extreme example of the persistent effect of iodine

medication was seen in a 55-year-old woman with a non-toxic goitre. An iodine solution in a dose of 5 drops daily was taken for a period of about one month; after treatment was stopped, the following serum iodine values were obtained: at five weeks, bound iodine 10·4 µg. per cent, unbound 2·8 µg. per cent, at seven weeks 9·8 and 2·2, at sixteen weeks 8·2 and 0·6, and at twenty-five weeks 5·6 and 1·2. A year later, the patient was asked to take 5 drops of saturated solution of potassium iodide daily for a period of one month. Before iodine administration the bound iodine was 6·3 µg. per cent, unbound 0·5 µg. per cent, and one week after the end of treatment 15·2 and 0·6, at seven weeks 9·9 and 1·5, at seventeen weeks the bound was 10·2 (unbound not determined), and at thirty weeks, 6·6 and 0·2. At all protein-bound iodine values above 8·0 µg. per cent, the radioactive iodine uptake was below 8 per cent. The uptakes with normal serum iodine values were 21 per cent, 28 per cent, and 37 per cent.

Transport Form of Bound Iodine

The findings of Gordon and collaborators (1952) that thyroxine in plasma is bound to a characteristic protein have been confirmed. This thyroxine-binding protein, a very small component not detectable by usual staining methods, migrates to a position near α_2-globulin upon zone electrophoresis. Its capacity for thyroxine can be measured by addition of thyroxine to plasma and determination of the proportion which is caused to spill over into the albumin zone. Capacity of the specific zone for thyroxine is taken as a measure of the quantity of thyroxine-binding protein. It would appear that the quantity of this protein in plasma bears a significant but imperfect relationship to the concentration of albumin, and it is a current concept that, other factors being equal, the concentration of protein-bound iodine in plasma varies directly with the amount of the specific carrier.

The low iodine in the serum in nephrosis (Recant and Riggs, 1952), cirrhosis of the liver (Kydd and Man, 1951), and in other states associated with profound hypoalbuminaemia

(Peters and Man, 1948) is probably to be accounted for by a deficiency of the specific carrier. Restoration of the albumin concentration toward normal by infusions of purified albumin did not cause a rise in the concentration of iodine but rather seemed to diminish it.

The increased quantity of bound iodine noted during pregnancy (Heinemann, Johnson and Man, 1948; Danowski, Gow, Mateer, Everhart, Johnston, and Greenman, 1950) has recently been shown to parallel closely an increase in the specific carrier (Dowling, Freinkel and Ingbar, 1956), changes which are reproducible in normal individuals by the administration of oestrogen. During the normal menstrual cycle of human beings no change in the iodine or proteins of serum has yet been detected (Danowski, Hedenburg and Greenman, 1949), but in the baboon a clear-cut cycle has been shown (Zyl, 1957*a*). Protein-bound iodine during menstruation and the early follicular phase of 3·8 µg./100 ml. rose to 4·9 µg./100 ml. in the late luteal phase and then fell again. Albumin showed a closely parallel cycle so that the iodine to albumin ratio remained constant. With pregnancy the rise during the luteal phase continued to a mean height of 5·42 µg./100 ml. which continued throughout pregnancy (Zyl, 1957*b*).

The diminished concentrations of bound iodine in association with decreased carrier protein and the increased values in pregnancy and following medication with oestrogen are unaccompanied by any clinical evidence of hypo- or hyperthyroidism. It might thus be inferred that normal quantities of thyroid hormone reach the tissues, and also that normal quantities of hormone are secreted from the thyroid gland. Diminished carrier protein must then favour entry of hormone into cells or action of hormone on cells, and it is of interest to inquire whether in Graves's disease it is an altered binding protein that accounts for the interesting findings of a more

condition (Hamolsky, Stein, and Freedberg, 1957).

In view of the large changes in protein-bound iodine that can be occasioned by changes in carrier protein, it is not unexpected that there should be such a wide range of values among normal individuals and a poor correlation between the severity of hyperthyroidism and the concentration of bound iodine in the serum. It is surprising in the light of the more rapid turnover of thyroxine in Graves's disease that there are not more instances of hyperthyroidism without increased protein-bound iodine in the serum.

Unusual Iodine Compound in Serum

Two years ago a patient with hyperthyroidism was found to have a radioiodine uptake of 96 per cent and the protein-bound iodine was greater than 20 µg./100 ml. A single dose of radioiodine brought about remission which was complete in less than a year. At intervals of two and twelve months after the initial observation the protein-bound iodine exceeded the limits of the test and the radioiodine uptakes were 100 per cent and 72 per cent respectively. Eighteen months after the therapy, quantitation showed the iodine to be 4,200 µg./100 ml. of serum, and six months thereafter, 3,100 µg./100 ml.

Meanwhile, other patients were encountered with similar findings, protein-bound iodine values of 2,000 to 18,800 µg./100 ml., normal or high radioiodine uptake, normal total urinary iodine, and no apparent source of exogenous iodine.

Examination of the case records of 104 patients showing values for bound iodine in excess of 32 µg./100 ml. (Fig. 1) disclosed that 75 had probably had radiographic media or some other form of iodine shortly before the blood samples were taken. In 29 cases, however, iodine ingestion did not seem a sufficient cause. From among these it was possible to obtain fresh samples of blood for analysis in 14 instances, and in all but 2 the protein-bound iodine was found to be in the very high range. It might be roughly estimated therefore that the incidence of these very high values was about 1 per cent of the total determinations shown in Fig. 1, and that about one-

quarter of the values above the limit of the method fell into this category.

At first it was incredible that such concentrations of an iodine compound could exist in plasma without altering iodine uptake by the thyroid and iodine excretion, and the possibility that some substance other than iodine might be catalytic in the ceric-arsenite reaction was considered. However, analysis by the Chaney method which involves distillation gave similar values; alkali incineration followed by thiosulphate titration to a starch end-point also gave similar values after correction for losses using radioiodine, and, finally, alkali fusion followed by oxidation by nitrous acid yielded material showing the typical iodine colour in chloroform.*

Isolation of the iodine-containing compound from the blood was accomplished by precipitating and washing the serum proteins with ethanol and extracting the diluted, acidified ethanol with chloroform. Purification was achieved by partition between chloroform and buffered aqueous alcohol, taking advantage of a favourable distribution into chloroform at pH 6 and into the aqueous phase at pH 8. The final alkaline extract was acidified to pH 3 and extracted with chloroform. After evaporation of the chloroform the green residue was leached repeatedly with boiling petroleum ether or hexane. Evaporation of the solvent left a colourless residue which on solution in a minimal volume of 40 per cent ethanol separated as needles upon addition of water or upon slow evaporation. Crystallization was facilitated by seeding; the first crystals to be obtained were derived from a petroleum ether solution on prolonged standing

The compound was readily detected on paper chromatograms by the method of Bowden, Maclagan and Wilkinson (1955); it precipitated with the serum proteins in trichloroacetic acid and migrated with albumin upon electrophoresis. It was not detectably labelled by oral tracer doses of radioiodine. The ultraviolet absorption spectrum resembled diiodotyrosine and thyroxine. In acid, the maximum at 294

* We are indebted to Dr. H. D. Purves for this demonstration.

in 50 per cent ethanol had an absorbance of 0·54/100 μg./ml. In alkali, the maximum was shifted to 318 (water) and 323 (ethanol) with absorbances of 1·2 and 1·26/1 mg./ml. respectively. Thus the maxima were intermediate between those of diiodotyrosine and thyroxine, but the increase in intensity upon solution in alkali more closely resembled diiodotyrosine.

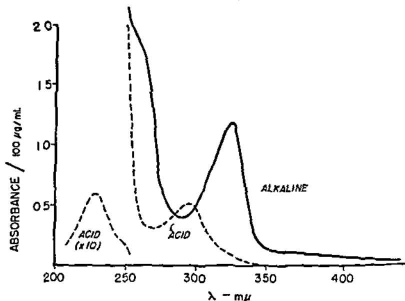


FIG. 6. Ultraviolet absorption spectrum of the unusual iodine compound isolated from serum. The position of the maximum at 296 and the shift to 322 upon addition of alkali as well as the degree of intensification of absorption were characteristic of o-diiodophenols

Shift of absorption in buffers indicated a pK similar to other o-diiodophenols. Titration of a 6 mg. sample with alkali using a glass electrode indicated an equivalent weight of approximately 274 and evidence of a carboxyl group. The m.p. was 150°, and analysis showed C, 23·65 per cent, H, 2·24 per cent, I, 64·39 per cent.*

The biological significance of this compound remains to be determined. No single feature characterized the 15 patients

* Dr. Martin Ettlinger provided guidance and helpful advice during this

thus far studied; 5 had hyperthyroidism, 5 had non-toxic goitre, and 5 showed no evidence of a thyroid disorder. Nine had had excessive iodine in some form months or years before, but 6 had had no exposure to iodine.

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REFERENCES

BARKER, S. B , HUMPHREY, M. J., and SOLEY, M. H (1951). *J. clin. Invest.*, 30, 55.
BOWDEN, C. H., MACLAGAN, N. F., and WILKINSON, J. H. (1955). *Biochem. J.*, 59, 93.
CHANEY, A. L (1940). *Industr Engng. Chem.* (Anal), 12, 179.
DANOWSKI, T. S , GOW, R. C., MATEER, F. M., EVERHART, W. C., JOHNSTON, S. Y., and GREENMAN, J. H. (1950). *Proc Soc. exp. Biol., N.Y.*, 74, 323.
DANOWSKI, T. S., HEDENBURG, S., and GREENMAN, J. H., (1949). *J. clin. Endocrin.*, 9, 768.
DANOWSKI, T. S., JOHNSTON, S Y , and GREENMAN, J. H. (1950). *J. clin. Endocrin* , 10, 519.
DOWLING, J T., FREINKEL, N., and INGBAR, S. H (1956) *J. clin. Endocrin Metab.*, 16, 1491.
GORDON, A. H., GROSS, J , O'CONNOR, D , and PITT-RIVERS, R. (1952). *Nature, Lond.*, 169, 19.
HAMOLSKY, M. W., STEIN, M , and FREEDBERG, A S. (1957). *J. clin. Endocrin Metab* , 17, 33.
HEINEMANN, M , JOHNSON, C. E., and MAN, E. B (1948) *J. clin Invest* , 27, 91.
[illegible] 914.
[illegible] Minnea-
[illegible]
PETERS, J P., and MAN, E. B. (1948). *J. clin. Invest.*, 27, 397.
RECANT, L., and RIGGS, D S (1952) *J. clin. Invest* , 31, 789.
[illegible] 9.
[illegible] *alyt.*
ZYL, A VAN (1957*a*) *J. Endocrin* , 14, 309.
ZYL, A. van (1957*b*) *J. Endocrin* , 14, 317.

DISCUSSION

Pitt-Rivers: Dr. Astwood, do you think that this compound is produced in the body or is it exogenous? Have you ever demonstrated its appearance, labelled, after the administration of radioiodine?

mination on this too, wouldn't it?

Astwood: It is being done.

Pitt-Rivers: An equivalent weight might be four times that, mightn't it? Has it got a sharp melting point?

Astwood: It melts very sharply at 150°.

Pitt-Rivers: It is rather low for the melting point of a thyroxine-like compound. The iododiphenyl ethers usually melt higher than that, unless they are hydrated or have solvent of crystallization. I don't think I am going to guess any more.

Astwood: The melting point is quite high for a diiodophenol.

Pitt-Rivers. For a diiodotyrosine derivative?

Astwood: Yes.

M. de Escobar: Do you know anything about the physiological activity of this compound?

Astwood No, I am afraid we do not.

Parkes: But you said this had no obvious relation to the thyroid condition, didn't you? It might be nothing of significance then

Astwood It is something!

phenolic group.

Astwood: The spectral shift with pH showed a pK of about 6·3, like other *o*-diiodophenols. But titration showed a stronger acid as well, which I assume to be a carboxyl

Morris: That would confirm that at least one iodine is near the phenolic residue.

Pitt-Rivers: Not one, there must be two because the pK of monoiodophenol is very much higher than that of diiodophenol.

Morris: Yes.

unforthcoming.

Astwood: I have a few patients that for two years have denied gallbladder radiograms or other sources of iodine.

It may be an ingredient of a new kind of facial cream or something.

Pitt-Rivers: Are they all females?

Astwood: All but one.

Ingbar: Have you had the opportunity to determine whether small amounts of inorganic iodine, the usual amounts, will produce the usual depression in iodine uptake in these patients?

Samuels: Have you tried any degradation reactions with the compound

it without being pretty sure what I was doing.

Samuels: Reactions which would split off side-chains to give you a benzoic acid type of derivative would be interesting to try; chromic acid, for instance.

I think it might be interesting to see whether you could use some

Astwood: Yes

Loraine. Have you attempted to correlate the PBI levels with TSH levels under normal and pathological conditions?

Astwood I am afraid not. McKenzie has really just started working on TSH in blood.

Ingbar In this survey of patients in whom you have done protein-bound iodine, have you encountered patients with bromide intoxication, and if so, what has been the effect on the measurement of PBI?

determination.

Astwood. I see.

Prunty: Have you any data on correlation between $PB^{131}I$ behaviour and PBI in serum in a thyrotoxic individual?

Astwood: Perhaps Prof. Prunty can answer this question for us! I haven't any figures myself.

Prunty: I have some data observed by Dr. J. S. Staffurth in our isotope laboratory. We have been trying to pay particular attention to the marginal cases of thyrotoxicosis, where the diagnosis is really in doubt. The problem is particularly accentuated in patients with nodular goitres. The data shows that in a group of patients with suspected Graves's disease, division into two fairly clear cut groups was possible. In one there was elevation of both PBI (above 7 5 µg./100 ml.) and $PB^{131}I$ (above 0 4 per cent of administered dose /l. plasma) but

Astwood: We haven't experience with $PB^{131}I$. It is the result of several variables and interpretation is difficult

M. de Escobar Dr. Astwood has spoken about his impression that in some of his goitre patients the PBI's were higher than one would expect to find. Were they patients from endemic areas?

Astwood: It may just be the result of my biased clinical acumen, but I have always looked for hyperthyroidism when seeing goitres, and perhaps I have seen it when I shouldn't have seen it Usually the protein-bound iodine has proved to be normal.

M. de Escobar: We have determined PBI's in patients with goitre from endemic areas of the province of Granada and we have also the same impression, that the PBI's were on the higher levels of the normal range. In some of the patients we were able to determine then the BEI and there was a rather big discrepancy between the values of the PBI and BEI. But we do not have enough cases to say anything conclusive on a statistical basis Do you think that in your cases it could be that the BEI is lower than the PBI?

operative patient is even more involved than that of a newly thyrotoxic individual.

Querido: There are two things I have heard mentioned this morning which were I think clinically extremely interesting, or could be interest-

ing. One is that Dr. Ingbar slipped some word about affinity of tissue for PBI; and the other was a remark that Dr. Astwood had some infor-

studied, myocardial slices, kidney, liver, there appear to be no striking differences between the tissue *per se.*

competitive basis Rather than keeping thyroxine out of the cell, TBP may carry thyroxine into the cell and may make its localization within the cell specific, perhaps directing it towards the site where the thyroxine would be metabolically active That is a possibility that I think we should bear in mind.

The group in Wisconsin has claimed that by making rats hyper-

fact that we have seen this sort of abnormality in "normal" relatives of hyperthyroid patients, who have never been thyrotoxic, as far as we could determine, at any time in the past

From the standpoint of interpretation of laboratory tests, one consequence of this persistent and abnormally rapid turnover of thyroxine is that the tissue utilization and tissue delivery of thyroid hormone may be excessive, though the PBI is within the normal range. A good number of other workers have observed this phenomenon.

Heller: I wonder whether you have considered whether circulatory changes in such patients play a rôle in these differences in uptake, changes which naturally you would not spot in your *in vitro* test?

Ingbar: That is entirely possible.

Ingbar: All I can say is that the function is not linear, and my comprehension of mathematics stops at linear functions!

Samuels: This would appear to indicate an absorption type of reaction, a removal from the equilibrium system.

Ingbar: Yes, one can readily reverse the uptake in the *in vitro* system. If one incubates tissue slices in saline one sees an asymptotically rising curve of thyroxine uptake by tissue. If one then adds a material which is rich in TBP, one extracts from the tissue a large share of the thyroxine which has been bound The cellular uptake then returns to approximately what it would have been if one had incubated in a protein medium in the first place.

Bush: In your measurements of disposal of thyroxine in these hyperthyroid patients, have you made any attempt to estimate metabolic products in the urine?

Ingbar No.

[*Note added in proof by Astwood*]

It was subsequently found that the iodine compound from blood closely resembled α-ethyl-3-hydroxy-2 : 4 : 6-triiodohydrocinnamic acid
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INSULIN IN BLOOD

P. J. RANDLE

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Methods of Assay

A NUMBER of assay procedures have been described which are sufficiently sensitive to be capable of detecting insulin in blood plasma. These are based upon the blood sugar response of adreno-demedullated alloxan-diabetic hypophysectomized rats (ADH rats) (Anderson, Lindner and Sutton, 1947), alloxan-diabetic hypophysectomized adrenalectomized rats (ADHA rats) (Bornstein, 1950), hypophysectomized alloxan-diabetic mice or rats (HAD mice or rats) (Beigelman *et al*, 1956*b*; Yenerman, Cornfield, Bates and Anderson, 1953); the increased uptake of glucose *in vitro* by the isolated rat diaphragm (Groen *et al.*, 1952; Willebrands and Groen, 1956; Vallance-Owen and Hurlock, 1954; Vallance-Owen, Hurlock and Please, 1955; Randle, 1954*c*, 1956*b*); and the gas output of slices of lactating mammary gland *in vitro* (Balmain *et al.*, 1954). Linear transformations of the dose-response relationship have been reported for each assay procedure. The minimum dose of insulin which may be detected by these procedures and the index of precision are given in Table I. The isolated rat diaphragm provides a simple and relatively inexpensive procedure for the assay of minute amounts of insulin and its suitability has been adequately confirmed. The precision is, however, not high The ADHA rat assay for insulin as originally described by Bornstein has not been confirmed. Peden (1955) has reported in detail the difficulties involved in the preparation of ADHA rats, and he was unable to obtain ADHA rats which were suitable for insulin assay. Randle (unpublished observations) has also attempted to prepare ADHA rats which were suitable for insulin assay. The yield of ADHA rats was low (only 119 out of 254 rats

Table I

PRECISION AND SENSITIVITY OF METHODS OF INSULIN ASSAY

Method of assay	*Minimum detectable quantity of insulin mu.*	*Index of precision*	*Authors*
Blood sugar response			
Alloxan-diabetic hypophysectomized adrenalectomized rats	0 05	Not known	Bornstein (1950)
Adreno-demedullated alloxan-diabetic hypophysectomized rats	0 125	Not known	Anderson *et al.* (1947)
Alloxan-diabetic hypophysectomized mice	0 00316	0 05–0 08	Yenerman *et al.* (1953)
Hypophysectomized alloxan-diabetic rats	2 5	0 30	Beigelman *et al.* (1956*a*, *b*)
	< 2	Not known	Randle (1954*a*)
Uptake of glucose *in vitro* by the isolated rat diaphragm	0·005	Not known	Groen *et al.* (1952)
	0 01	Not known	Vallance-Owen and Hurlock (1954)
	0 10	0 20–0 40	Randle (1954*a*; 1956*b*)
Gas output *in vitro* by slices of mammary gland from lactating rats	2	0 3	Balmain *et al.* (1954)

survived the preparative procedure) and the survival was very poor (mean survival time 7·3 days.) Those rats which did survive the preparative procedure were found to be too delicate and to possess too unstable a blood sugar to permit their use for insulin assay. The remaining procedures based upon blood sugar response have received some confirmation, though there is some disagreement as to the sensitivity to insulin of HAD rats (Table I). The assay based upon the gas output of slices of lactating mammary gland has not been much studied, nor has it, unlike the other procedures, been applied to the insulin content of plasma, but it appears to offer great promise.

The procedures based upon the ADHA rat and the isolated

rat diaphragm are those which have been most extensively applied to the assay of plasma insulin. Since it has been shown that plasma may contain factors other than insulin which exert an effect in these assay procedures, the results obtained with the ADHA rat and rat diaphragm do not necessarily reflect the insulin content of plasma. In this account of the subject, the term plasma insulin activity is therefore used to describe the insulin-like action of plasma in a bioassay without implying that the action is solely that of insulin in the plasma.

The Insulin Activity of Plasma from Normal Animals

The insulin activity of plasma from normal people has been determined by a number of procedures and the results are summarized in Table II. The values reported vary widely. Bornstein (1950) using the ADHA rat found activity

Table II

INSULIN ACTIVITY OF PLASMA FROM NORMAL ANIMALS

Species	*Method of assay*	*Plasma insulin activity mu /ml.*	*Author*
Man	ADHA rat	0 10 (fasting) 0 20 (1 hr. after oral glucose) 0 34 (2–2 5 hr after oral glucose)	Bornstein (1950)
	ADHA rat	2 (acid alcohol extract of plasma)	Weitze and Hagedorn (1954)
	HAD rat	< 2	Randle (1954c)
	Rat diaphragm	0 1 to 3 0 01 to 0 10 (fasting)	Willebrands and Groen (1956)
		0 10 to 0 80 (1 hr after oral glucose)	Vallance-Owen and Hurlock (1954)
		1 to 20 (fasting or 2–2 5 hr after oral glucose)	Randle (1954c)
Rat	Rat diaphragm	5 to 30 (fasting)	Randle and Young (1956)
Cat	Rat diaphragm	2 to 3	Randle and Young (1956)

mu = milliunits

equivalent to 0·1 mu. insulin/ml. in plasma from fasting normal people rising to 0·2 mu./ml. and 0·34 mu./ml. one and two hours, respectively, after oral glucose. Willebrands and Groen (1956) using the isolated rat diaphragm give values of 0·1 to 3 mu./ml. plasma. Using a similar procedure Vallance-Owen and Hurlock (1954) find 0·01–0·1 mu./ml. of plasma from fasting normal people and 0·1 to 0·8 mu./ml. one hour after oral glucose, while Randle (1954c, and unpublished observations) finds activity equivalent to 1–20 mu./ml. plasma from normal people 2–2·5 hours after oral glucose, with similar values for plasma obtained from fasting people.

Weitze and Hagedorn (1954) and Beigelman and co-workers (1956a) have used a somewhat different approach—by attempting to extract insulin from plasma prior to assay. Weitze and Hagedorn extracted plasma from normal people with acid alcohol and assayed the resultant extract for insulin by the blood sugar response of ADH rats, finding activity equivalent to 2 mu. insulin/ml. plasma. Beigelman and co-workers (1956a) have tested plasma protein fractions for insulin activity by the blood sugar response of HAD rats. Although these experiments were largely qualitative, their results suggest that the insulin activity of normal human plasma may be as high as 2 mu./ml.

These assays were for the most part carried out with single doses of plasma. Bornstein assayed only one dose of plasma and did not incorporate standard doses of insulin in the assay, and the assumption was made in calculating plasma insulin activity that the response to insulin was constant from assay to assay. Four- or six-point assays were not made. In most of the assays using the rat diaphragm only one concentration of plasma was tested, though standard doses of insulin were included in all assays. Groen and collaborators (1952) have carried out assays on plasma diluted fivefold with a balanced salt solution (Gey and Gey, 1936) prior to assay: [illegible] Owen and Hurlock (1954) have [illegible]
while [illegible]
Gey's

Table III

FOUR-POINT ASSAYS OF INSULIN ACTIVITY OF NORMAL HUMAN PLASMA BY THE GLUCOSE UPTAKE OF THE ISOLATED RAT DIAPHRAGM *IN VITRO*

Plasma sample	*Glucose uptake mg. glucose/g. wet diaphragm/hr. incubation Mean ± S.E. of mean in the presence of*						*Calculated plasma insulin activity mu./ml.*	
	Plasma			*Insulin*				
	Undiluted	*Fourfold dilution*	*Difference (A)*	*4 mu./ml.*	*1 mu./ml.*	*Difference (B)*	*Undiluted*	*Fourfold dilution*
1	4·33 ± 0·18	3·38 ± 0·15	0·95 ± 0·25	4·01 ± 0·18	3·17 ± 0·17	0·84 ± 0·25	0·7	5·0
2	2·95 ± 0·16	2·91 ± 0·19	0·04 ± 0·21	4·10 ± 0·13	2·73 ± 0·11	1·37 ± 0·21	1·2	4·8
3	2·63 ± 0·13	2·50 ± 0·11	0·13 ± 0·19	3·89 ± 0·18	2·90 ± 0·12	0·99 ± 0·10	0·7	2·3
4	2·15 ± 0·15	2·55 ± 0·10	−0·40 ± 0·17	4·04 ± 0·13	2·74 ± 0·10	1·30 ± 0·17	0·5	3·3
5	2·83 ± 0·22	2·54 ± 0·10	0·29 ± 0·20	4·43 ± 0·25	2·72 ± 0·20	1·71 ± 0·20	1·1	3·5

Each value given for glucose uptake is the mean of six observations.

Significance of differences. A.—sample 1, $P < 0·01 > 0·001$; samples 2–5, $P \gg 0·05$.
B.—sample 1, $P < 0·01 > 0·001$, samples 2–5, $P < 0·001$.

Significance of differences (B − A): sample 1, $P \gg 0·05$.
samples 2–5, $P < 0·01$.

In the assay of sample 5, plasma was diluted fivefold and the lower dose of insulin was 0·8 mu./ml.

Four-point assays with the rat diaphragm employing normal human plasma diluted 16-fold and fourfold and insulin (0·5 and 2 mu./ml.) have been reported (Randle, 1954c). The slopes of regression lines for plasma and insulin did not differ significantly under these conditions.

Four-point assays using normal human plasma (undiluted and fourfold dilution) and insulin (4 and 1 mu./ml.) have recently been made with the isolated rat diaphragm. The results are summarized in Table III. With one exception the changes in response to fourfold changes in the concentrations of insulin and plasma differed significantly. An increase in the concentration of insulin always produced a significant increase in glucose uptake, whereas increase in the concentration of plasma under these conditions did not produce an increase in glucose uptake, in most experiments (Table III). Similar results have been obtained in six-point assays with plasma from fasted normal rats (unpublished observations). It is to be inferred from this observation that the insulin activity of undiluted plasma would be lower than that of diluted plasma (cf. Table III). This observation would explain in part the difference between the values for insulin activity of normal human plasma reported by Vallance-Owen and Hurlock and by Randle.

The results summarized in Table II do not yield an acceptable estimate of the insulin content of normal human plasma. The wide variation in results, even where essentially the same assay procedure has been employed, and the unsatisfactory results obtained where four- or six-point designs have been used, suggest that the methods of assay are not specific when applied to the insulin content of plasma from normal animals.

Plasma Insulin Activity in Diabetes

The insulin activity of plasma from rats exhibiting severe alloxan diabetes is substantially lower than that of normal rat plasma when estimated by the glucose uptake of the isolated rat diaphragm (Tuerkischer and Wertheimer, 1948; Park and Bornstein, 1953; Randle, 1955; Whitney and Young, 1957).

The insulin activity of plasma from alloxan-diabetic hypophysectomized rats appears to be normal, but may be reduced to the low level characteristic of alloxan-diabetic rats by treatment with growth hormone and cortisone (Park and Bornstein, 1953; Whitney and Young, 1957). Park and Bornstein (1953) have inferred that the low insulin activity of plasma from alloxan-diabetic rats is a manifestation of an inhibitor in the plasma, and inhibitory material, which may be a lipoprotein, was separated from the plasma by fractionation (Bornstein, 1953). Randle (1955) observed that low insulin activity of plasma from alloxan-diabetic rats was only a feature of fresh plasma and that plasma which had been repeatedly frozen and thawed possessed normal insulin activity. This observation would be in keeping with the conclusions of Park and Bornstein, since freezing and thawing were reported to inactivate their inhibitor.

Plasma from totally depancreatized cats and dogs deprived of insulin is devoid of insulin activity when tested by the rat diaphragm method, but insulin activity may be restored to normal by treatment with insulin (Groen *et al.*, 1952; Randle, 1956*a*.) Insulin activity is not restored to the plasma of depancreatized cats deprived of insulin when the plasma is frozen and thawed, and an inhibitor of the type identified in the plasma of alloxan-diabetic rats is thus unlikely to be present (unpublished observations). However, the plasma of depancreatized cats may contain an inhibitor, not inactivated by freezing and thawing, since Vallance-Owen (personal communication) finds that plasma from such animals inhibits the action of insulin added *in vitro* upon the glucose uptake of the isolated rat diaphragm.

The insulin activity of plasma from cases of human diabetes has been studied by assays based upon the ADHA rat and the isolated rat diaphragm. Bornstein and Trewhella (1950) and Bornstein and Lawrence (1951) find that plasma from untreated cases of severe spontaneous diabetes presenting with weight loss and ketonuria is devoid of insulin activity, but that insulin activity in essentially normal amounts can be

detected in the plasma after treatment with insulin. Plasma from untreated cases of milder diabetes who presented with obesity and without ketonuria possessed insulin activity, which was somewhat lower than that of normal human plasma. Plasma from untreated cases of severe diabetes, in addition to being devoid of insulin activity, inhibited the action of insulin, measured in a subsequent test, on the blood sugar level of ADHA rats.

Groen and co-workers (1952), who examined the insulin activity of plasma from the types of human diabetic mentioned previously by means of the isolated rat diaphragm, found essentially normal insulin activity in the plasma, though plasma from cases of severe untreated diabetic acidosis was devoid of insulin activity. Similar results have been obtained by Randle (unpublished observations). Vallance-Owen, Hurlock and Please (1955) find that plasma from cases of severe spontaneous diabetes which have been treated with insulin, but in whom the diabetes is poorly controlled, is devoid of insulin activity and contains a factor which inhibits the action of insulin on the uptake of glucose by diaphragm. In similar cases in which diabetes was well controlled the plasma insulin activity was essentially normal and no inhibitor could be detected in the plasma. In untreated cases of milder diabetes with obesity the plasma insulin activity was normal, or under some conditions above normal. The apparent discrepancy between the results of Vallance-Owen and collaborators and those of Groen and co-workers and of Randle in severe cases of diabetes may result from the concentration of plasma used in the assay of plasma insulin activity. Thus Vallance-Owen, Hurlock and Please have used undiluted plasma, while Groen and co-workers and Randle have used plasma diluted prior to assay. The inhibitor which Vallance-Owen, Hurlock and Please have noted in the plasma of poorly controlled cases of diabetes might well exert a greater effect on the uptake of glucose by diaphragm in undiluted plasma.

It may be inferred from these observations that the insulin content of plasma from diabetic animals is not known. In the

instances in which insulin activity of diabetic plasma has been found to be low, the activity of inhibitors in the assay system has been demonstrated. The observation that the plasma of alloxan-diabetic rats, in which the inhibitor has been inactivated, possesses essentially normal insulin activity by the rat diaphragm method does not necessarily mean that the insulin content of the plasma is normal, since it is possible that the insulin-like action of plasma upon the glucose uptake by diaphragm is in part an effect of plasma proteins other than insulin (cf. pp. 128–129).

The Influence of the Pituitary on Plasma Insulin Activity

The influence of pituitary insufficiency on plasma insulin activity has been studied by the rat diaphragm method. Randle (1945*b*) observed a marked reduction of plasma insulin activity in severe cases of panhypopituitarism in man, and Randle and Young (1956) found that the insulin activity of plasma from hypophysectomized rats was substantially less than that of plasma from normal rats. These assays were carried out on plasma diluted fourfold, prior to assay, with Gey's balanced salt solution. When assays of insulin activity are carried out on undiluted plasma from hypophysectomized rats, insulin activity can be detected which in some instances is comparable to that of undiluted normal rat plasma. The insulin activity of plasma from hypophysectomized rats is therefore not necessarily lower than that of normal rat plasma.

The reduced insulin activity of diluted plasma from hypophysectomized rats may result from the presence in the plasma of some inhibitory material. Protein fractions were prepared from the plasma of hypophysectomized rats by the method of Ulrich, Li and Tarver (1954) The fractions obtained were:

Fraction I	precipitated from plasma at pH 7·2 and 8 per cent ethanol concentration.
Fraction II	precipitated from supernatant from fraction I at pH 7·0 and 20 per cent ethanol concentration.

Fraction III precipitated from supernatant from fraction II at pH 5·7 and 40 per cent ethanol concentration.

Fraction IV precipitated from supernatant from fraction III at pH 4·8 and 40 per cent ethanol concentration.

Fractions II–IV after dialysis (against distilled water) and lyophilization were reconstituted with Gey's balanced salt solution to their concentration in original plasma diluted fourfold. They were then compared for insulin activity with a sample of the plasma from which they had been prepared and which was diluted fourfold prior to assay. The results are summarized in Table IV. Fractions II and III, unlike

Table IV

INFLUENCE OF PLASMA, AND PROTEIN FRACTIONS OF PLASMA FROM HYPOPHYSECTOMIZED RATS UPON THE UPTAKE OF GLUCOSE *IN VITRO* BY THE ISOLATED RAT DIAPHRAGM

Addition to buffer	*Uptake of glucose. Mean ± S.E. of mean mg. glucose/g. wet diaphragm/hr. incubation*
No addition (buffer alone)	(*a*) 1 87 ± 0 05
Plasma	(*b*) 1·95 ± 0·11
Fraction II	(*c*) 2 47 ± 0·11
Fraction III	(*d*) 2 46 ± 0·12
Fraction IV	(*e*) 2 07 ± 0·12

[illegible] The plasma was a pooled sample [illegible]

Significance of difference between means:
(*b–a*), (*e–b*): $P \geqslant 0{\cdot}05$; (*c–b*), (*d–b*): $P < 0\ 01 > 0{\cdot}001$; (*e—c*), (*e–d*): $P < 0{\cdot}05 > 0{\cdot}01$.

fraction IV and the original plasma, stimulated the glucose uptake by rat diaphragm. These observations would suggest that plasma from hypophysectomized rats contains a factor

which inhibits the uptake of glucose by rat diaphragm *in vitro*, but an inhibitor has not so far been identified or detected in plasma fractions.

The influence of treatment with growth hormone on plasma insulin activity has been studied with the ADHA rat and the isolated rat diaphragm. Bornstein, Reid and Young (1951) found that the portal vein blood of intact cats treated with growth hormone for several days contained a substance which was hyperglycaemic in the ADHA rat. Femoral vein blood was devoid of hyperglycaemic activity, and insulin activity could be detected in some instances. Bornstein and Lawrence (1951) reported that plasma from acromegalic patients with untreated diabetes was also hyperglycaemic in the ADHA rat. Since the activity of hyperglycaemic material would be expected to prevent the detection of insulin activity by the ADHA rat these observations provide no definite information about the influence of growth hormone on plasma insulin activity in the cat and in man. Randle and Young (1956) have used the isolated rat diaphragm to study the influence of treatment with growth hormone on plasma insulin activity in the cat and rat. In the intact cat, treatment for several days with growth hormone led to a substantial increase in plasma insulin activity. When totally depancreatized cats were treated with insulin and with similar amounts of growth hormone, the insulin activity of the plasma was unaltered unless additional insulin was supplied during the period of treatment with growth hormone. Randle and Young concluded that treatment with growth hormone leads to a rise in the insulin content of the plasma. Plasma from a cat rendered permanently diabetic by prolonged treatment with growth hormone (metahypophysial diabetes) contained a factor which inhibited the uptake of glucose by rat diaphragm. When intact rats were treated with growth hormone for several days the insulin activity of the plasma was unaltered —a result in clear contrast to that obtained in the cat. The insulin activity of plasma from many acromegalic patients was found to be greater than that of normal human plasma

(Randle, 1954*a*, 1955), an observation which has been confirmed (Willebrands and Groen, 1956; Candela, 1956). The insulin activity of plasma from acromegalic patients may revert to normal levels after pituitary irradiation (Randle, 1955).

The influence of other pituitary hormones on plasma insulin activity has not been studied.

These observations provide little direct evidence for an influence of the pituitary on the plasma level of insulin. The observations of Randle and Young would suggest that in the cat treatment with growth hormone leads to an increase in plasma insulin content. This conclusion was, however, drawn from a comparison of the effects of growth hormone in intact and depancreatized cats and was not based upon the assumption that the method of assay employed was specific for insulin in plasma. Bennett (1955) and Milman, de Moor and Lukens (1951) using indirect criteria have also obtained evidence which would suggest that treatment with growth hormone in the intact cat or dog leads to an increase in plasma insulin content.

Plasma Insulin Activity and Spontaneous Hypoglycaemia

Plasma insulin activity has been estimated in human cases of spontaneous hypoglycaemia by the rat diaphragm method. Plasma insulin activity appears to be above normal in spontaneous hypoglycaemia associated with islet cell tumour of the pancreas (Groen *et al.*, 1952; Willebrands and Groen, 1956; Vallance-Owen, personal communication), though this does not appear to be so for all samples of plasma obtained in such cases (unpublished observations). Plasma insulin activity apparently returns to normal levels following surgical removal of the tumour (Vallance-Owen, personal communication). Plasma insulin activity appears to be normal in cases of functional hypoglycaemia (Groen *et al.*, 1952), but may be enhanced in some cases of familial hypoglycaemia in children (unpublished observations).

Insulin Activity of Plasma Protein Fractions

Beigelman and co-workers (1956a) have tested, for insulin activity, protein fractions prepared from normal human plasma by blood plasma fractionation methods developed by Cohn and his collaborators. The plasma fractions were tested for insulin activity by the blood sugar response of HAD rats. Insulin activity appeared to be associated with certain β-globulins and lipoproteins and was not identified in other plasma protein fractions.

The influence of protein fractions of plasma upon the glucose uptake of the isolated rat diaphragm has been little studied. Both albumin and globulin fractions prepared from the plasma of acromegalic patients and normal rats appear to be capable of stimulating glucose uptake, though the γ-globulin fraction of plasma from an acromegalic patient lacked stimulating activity (unpublished observations).

Specificity of Methods of Assay

Procedures for the assay of insulin in blood plasma can only be considered useful if the insulin-like action of plasma in the bioassay is due solely to an action of insulin in the plasma. Such a procedure may be considered quantitative if it can be shown that plasma is devoid of substances which inhibit or potentiate the action of insulin in the bioassay. Not one of the assay procedures discussed here has so far been shown to be quantitative when applied to the insulin content of plasma. Thus four- or six-point assays have not been carried out with methods of assay based upon blood sugar response. In the case of the isolated rat diaphragm four- and six-point assays with plasma from normal animals have revealed significant differences between the quantitative relationship of dose to response for plasma and for insulin. In some instances plasma has been shown to contain substances which interfere with the action of insulin in the bioassay. Thus plasma from diabetic animals has been shown to inhibit the action of insulin on the blood sugar response of ADHA rats and the uptake of glucose by the isolated rat diaphragm.

Furthermore, some samples of plasma from acromegalic patients or from intact cats treated with growth hormone have been shown to be hyperglycaemic in the ADHA rat. Thus these procedures cannot be considered specific when applied to the insulin content of untreated plasma.

The insulin-like action of plasma upon the uptake of glucose by rat diaphragm has been variously attributed to the activity of insulin in the plasma (Groen *et al.*, 1952; Vallance-Owen and Hurlock, 1954) or to a non-specific effect of plasma protein (Park and Bornstein, 1953). In support of the former view it has been shown that plasma from depancreatized animals is devoid of insulin activity unless the animals are treated with insulin and that the insulin activity of normal human plasma, like that of insulin, is abolished by treatment with cysteine (Groen *et al.*, 1952; Randle, 1954*c*, 1956*b*; Vallance-Owen and Hurlock, 1954). Park and Bornstein find that, although plasma from alloxan-diabetic rats is devoid of insulin activity, plasma from alloxan-diabetic hypophysectomized rats possesses normal insulin activity. They attribute the low activity of plasma from alloxan-diabetic rats to an inhibitor, which is not present in the plasma of alloxan-diabetic hypophysectomized rats. They have further shown that a 3 per cent solution of crystalline bovine serum albumin is capable of stimulating the uptake of glucose by diaphragm and that the inhibitor (present in alloxan-diabetic rat plasma) is capable of preventing or reducing the stimulating effect upon glucose uptake both of serum albumin and of insulin. It may be inferred from these observations that the low insulin activity of plasma from diabetic animals may result from the activity of an inhibitor which prevents a non-specific effect of plasma protein and not from the low insulin content of the plasma. Randle (1954*b*) and Randle and Young (1956) have observed that samples of plasma from cases of panhypopituitarism and from hypophysectomized rats do not stimulate the uptake of glucose by diaphragm. Since plasma from cases of panhypopituitarism and from hypophysectomized rats did not stimulate glucose uptake, it was concluded that

insulin-like action of plasma from normal animals was unlikely to be due to a non-specific effect of plasma protein; since it was to be expected that plasma from hypopituitary animals would be free from inhibitors of glucose uptake (Randle, 1954*b*; 1955). More recent observations presented here suggest that the lack of an insulin-like action of plasma from hypophysectomized rats upon glucose uptake is also due to the presence in the plasma of a factor which inhibits glucose uptake and so masks the stimulating effect of the plasma.

The evidence presented here would thus suggest that the stimulating action of plasma on glucose uptake results at least *in* part from a non-specific effect of plasma protein. Nevertheless the evidence is not conclusive, for crystalline serum albumin and diabetic serum might contain sufficient insulin

Table V

INFLUENCE OF OVALBUMIN, AMANDIN AND CRUDE RAT SERUM ALBUMIN *IN VITRO* UPON THE UPTAKE OF GLUCOSE BY THE ISOLATED RAT DIAPHRAGM

Experiment No	*Addition to buffer*	*Glucose uptake, mean ± S E. of mean mg. glucose/g wet diaphragm/hr. incubation*	*Significance of difference between means*
1	None	3 31 ± 0 34 (*a*)	$(d-a)$ $P < 0\ 02 > 0\ 01$
	Ovalbumin	2 95 ± 0 24 (*b*)	$(d-b)$ $P < 0\ 01 > 0\ 001$
	Amandin	3 61 ± 0 20 (*c*)	$(d-c)$ $P < 0\ 05 > 0\ 02$
	Crude serum albumin	5 12 ± 0 54 (*d*)	other $P \gg 0\ 05$
2	None	3 00 ± 0 17 (*a*)	$(b-a)$ $P < 0\ 02 > 0\ 01$
	Ovalbumin	4 11 ± 0 29 (*b*)	$(c-a)$ $P < 0\ 01 > 0\ 001$
	Amandin	4 43 ± 0 33 (*c*)	$(d-b)$ $P < 0\ 01 > 0\ 001$
	Crude serum albumin	5 79 ± 0 42 (*d*)	$(d-c)$ $P < 0\ 05 > 0\ 02$
3	None	1 63 ± 0 08 (*a*)	$(d-a)$ $P < 0\ 02 > 0\ 01$
	Ovalbumin	1 81 ± 0 06 (*b*)	$(d-b)$ $P < 0\ 05 > 0\ 02$
	Amandin	1 67 ± 0 10 (*c*)	$(d-c)$ $P < 0\ 05 > 0\ 02$
	Crude serum albumin	2·34 ± 0 23 (*d*)	other $P > 0\ 05$

Each mean was derived from 6 observations

Proteins were prepared and tested as 1 5 per cent solutions.

to account for their stimulating activity. Thus a contamination of serum albumin with 1 part of insulin to 10^6 parts of serum albumin would be sufficient to account for its stimulating activity. In view of this we have recently tested for stimulating activity two proteins which should be free of insulin—amandin, a crystalline globulin obtained from almonds, and crystalline ovalbumin. The proteins were tested as a 1·5 per cent solution in Gey's balanced salt solution and compared with a 1·5 per cent solution of crude rat serum albumin. In one of three experiments a small but nevertheless significant stimulation of glucose uptake was observed with amandin and ovalbumin. Crude rat serum albumin was, however, effective in stimulating glucose uptake in all experiments and exerted a substantially greater effect than the other proteins (Table V).

In the case of assays based upon blood sugar response the possibility that substances in plasma, other than insulin, might exert an hypoglycaemic effect does not appear to have been considered.

General Conclusions

In the present state of knowledge of the subject of insulin in blood, it would appear that although there are now available a number of highly sensitive methods for the assay of insulin, there is no method of assay which can be satisfactorily applied to the insulin content of untreated plasma. Thus the development of a method for the specific assay of insulin in blood plasma involves either the development of a more specific method of bioassay or the development of methods for the extraction of insulin from plasma, prior to assay. The recent report by Beigelman and collaborators (1956*a*, *b*) upon the insulin activity of plasma protein fractions would appear to offer interesting possibilities in this connection. Amongst biological responses to insulin which do not appear to have been used for bioassay, the glucose uptake of the perfused isolated heart appears promising (Bleehan and Fisher, 1954).

The results which have been obtained with methods which

are generally conceded to be of doubtful specificity suggest that the insulin content of normal human plasma is of the order of 0·1 to 10 mu. insulin/ml. plasma. The insulin content of the blood appears to be lower in fasting animals than in animals fed with glucose. The plasma insulin content appears also to be increased in cases of spontaneous hypoglycaemia in man associated with β-cell tumours of the pancreatic islets, in cases of acromegaly, and in cats and dogs following treatment with pituitary growth hormone. The insulin content of plasma from diabetic patients and animals seems uncertain, since the presence of insulin antagonists in plasma from such animals may prevent the assay of insulin in the plasma.

Acknowledgements

REFERENCES

ANDERSON, E., LINDNER, E., and SUTTON, V. (1947) *Amer J. Physiol*, 149, 350

BALMAIN, J. H, COX, C P., FOLLEY, S. J., and MCNAUGHT, M. L. (1954). *J. Endocrin.*, 11, 269

[illegible]

BENNETT, L L. (1955) *In* The Hypophyseal Growth Hormone, Nature and Actions, p 447. New York: McGraw-Hill.

BLEEHAN, N. M, and FISHER, R B (1954). *J. Physiol*, 123, 260.

BORNSTEIN, J. (1950). *Aust. J. exp. Biol med. Sci.*, 28, 87, 93.

BORNSTEIN, J. (1953). *J. biol. Chem.*, 205, 513.

BORNSTEIN, J., and LAWRENCE, R. D. (1951). *Brit. med. J.*, 2, 1541.

BORNSTEIN, J, REID, E., and YOUNG, F. G. (1951). *Nature, Lond.*, 164, 209.

BORNSTEIN, J., and TREWHELLA, P (1950) *Aust. J. exp. Biol. med. Sci.*, 28, 569.

CANDELA, R (1956). Ciba Foundation Colloquia on Endocrinology, 9, 51 London: Churchill.

GEY, G O, and GEY, M. K. (1936). *Amer. J. Cancer*, 27, 45.

GROEN, J, KAMMINGA, C. E., WILLEBRANDS, A. F., and BLICKMAN, J R. (1952). *J. clin. Invest*, 31, 97.

MILMAN, A. E., DE MOOR, P., and LUKENS, F. D. W. (1951). *Amer. J. Physiol.*, 140, 08.
PARK, C R, and BORNSTEIN, J. (1953). *J. biol. Chem.*, 205, 503.
PEDEN, A. S. (1955). *Acta Endocr, Copenhagen*, 18, 67.
RANDLE, P. J. (1954*a*). *Lancet*, 1, 441.
RANDLE, P. J. (1954*b*). *Lancet*, 1, 809
RANDLE, P. J. (1954*c*). *Brit. med J.*, 1, 1232.
RANDLE, P. J. (1955) *In* The Hypophyseal Growth Hormone, Nature and Actions, p. 143 New York McGraw-Hill.
RANDLE, P. J. (1956*a*) Ciba Foundation Colloquia on Endocrinology, 9, 35. London: Churchill.
RANDLE, P. J. (1956*b*). *J Endocrin.*, 14, 82.
RANDLE, P. J., and YOUNG, F. G (1956). *J. Endocrin.*, 13, 335
TUERKISCHER, E., and WERTHEIMER, E. (1948). *Biochem. J.*, 42, 603.
ULRICH, E, LI, C. H., and TARVER, H. (1954). *Arch Biochem. Biophys*, 50, 421.
VALLANCE-OWEN, J., and HURLOCK, B. (1954) *Lancet*, 1, 68.
VALLANCE-OWEN, J., HURLOCK, B., and PLEASE, N. (1955) *Lancet*, 2, 583
WEITZE, M., and HAGEDORN, H. C. (1954). *In* Experimental Diabetes, C.I.O.M S. Symposium, p. 279. Oxford: Blackwell.
WHITNEY, J. E, and YOUNG, F G (1957) *Biochem J* In press.
WILLEBRANDS, A F., and GROEN, J. (1956). *Diabetes*, 5, 378.
YENERMAN, M., CORNFIELD, J., BATES, R W., and ANDERSON, E (1953). *Fed Proc*, 12, 162

DISCUSSION

Vallance-Owen: This question of the antagonism to insulin in plasma has been exercising us for some time. We have used undiluted plasma throughout because we felt that we were dealing with a delicate situation of insulin on the one hand and its antagonists on the other, and that we therefore would be measuring the effective insulin concentration We first noticed the antagonism to insulin in our study of diabetic patients.

We were particularly interested in the two broad clinical types of diabetics—those who need insulin to live, without which they rapidly lose weight and become ketotic; and those who do not need insulin and who have no tendency to ketosis unless their diabetes is complicated by infection. These patients are usually obese and often recover from the diabetic state when they lose weight on a low carbohydrate diet. All I will say about these mild obese patients is that we have found insulin activity in their plasma, essentially in the normal range

However, when we came to study the uncontrolled insulin-requiring diabetics, but who were not necessarily ketotic, we found no insulin activity in their plasma, and moreover when we added insulin to the plasma from these patients its activity was inhibited. These patients were having up to but usually less than 100 u. insulin daily, and this by definition is not insulin resistance; they showed no allergic reactions. Perhaps more important, several new patients were examined who sub-

Table I summarizes the results we have obtained The mean glucose

Table I (Vallance-Owen)

Group and (No.)	*Mean glucose uptake above the level of diaphragm in buffer alone (±S E M.) mg. per 100 ml per 10 mg. diaphragm*		
	*Buffer + insulin**	*Plasma alone*	*Plasma + insulin**
Normal (7)	12 63±0·74	6 60±0·73	13 33±0 82
Depancreatized (9)	*11 73±0·50*	*0 57±0 48*	*6 19±0 47*
Depan. hypox (3)	11 60±0 68	0 20±0·09	11 10±0 75
Depan. hypox+F (3)	11 55±0 74	0 14±0 32	12 25±0 63
Depan hypox.+GH (4)	11 33±0·35	0 28±0 52	11 95±1 05
Depan adrex. (3)	12 53±0 27	0 81±1 03	13 22±0 81
Depan. adrex+E or F (3)	11 52±0 64	−0 50±0 89	2 57±1 92
Depan adrex+GH (3)	11 80±0 79	−0 50±0 58	11 60±0 82

* Insulin was added to make 1000 μu per ml buffer or plasma

make a final concentration of 1000 μu/ml In the case of the depancrea-

Now we have found that if cortisone or cortisol was injected for 4 days into these Long-Lukens animals we could restore the inhibiting properties to their plasma (line 7), but when we injected the same dose of

us, I am afraid, to carry out the final combination of growth hormone and cortisone in the Houssay animal. However, our conclusions are that the pituitary and adrenal oxysteroids must be present for insulin antagonism to be found in the plasma from depancreatized cats.

Randle: Dr. Vallance-Owen's results strengthen the idea, first put forward by Bornstein and Park, that a substance capable of inhibiting the action of insulin on the glucose uptake of the isolated rat diaphragm *in vitro* is found in the serum of diabetic animals, but only when both growth hormone and an adrenal steroid are available. We do not know yet whether this is formed from or under the influence of growth hormone Dr. Vallance-Owen's results introduce one discrepancy. Born-

much the same kind of scale as Dr. Randle with a similar lack of success

animals we used. However, we went on to use various other colonies of rats, and in an additional 84 experiments, we found very significant increases in glucose uptake produced by quantities of insulin of the order of 10^{-5} u./ml. Out of those 84 experiments, something like 57 were

6×10^{-6}; the index of precision was 1·37. We concluded that, certainly in our experience, it was impossible to get an accurate assay. We also carried out many experiments with plasma

card these anomalous glucose uptakes we felt this to be unsatisfactory and have abandoned this technique Certainly with our own animals, we would need to use such large numbers to obtain a valid assay that it would be impossible for the department to cope.

I have had an opportunity of seeing Dr. Randle's figures, and there is no doubt that his index of precision and his slopes are very much better than ours. I haven't seen in detail the results of Dr. Vallance-Owen, but I just do feel this discrepancy between the results in the normal individual might be due to this variation in slope and the fact that not enough animals are used.

Did Dr Randle and Dr. Vallance-Owen also have bad days when the slope was zero and if so what did they do? I have been in touch with another laboratory that has had a similar experience to ours, namely, first of all they had insensitive animals, and then when they got sensitive animals there wasn't much difference between the effects of 10^{-3} and 10^{-6} u. insulin

many other workers who have reported on the glucose uptake of the

think that this discrepancy is in any way connected with errors of the

plasma

Gray: I feel fairly sure that there is a strain difference and we have just been unlucky with our rats, but there are, as you say, quite a number of other places where they have not had any luck either.

Vallance-Owen: Well, we carried the method over to Dr. Lukens and Dr. Stadie, and using the American rats found that the slopes of the lines were similar to those achieved in Hammersmith. Several workers from various laboratories came to study the method and I have heard from them subsequently. They say that although it took them a month or two to iron out the gimmicks they are now getting satisfactory lines.

Gray: Even when the gimmicks have been ironed out, do they get bad days?

Vallance-Owen: That I don't know.

Gray How many diaphragms do you use?

Vallance-Owen: Three or four for each point.

Gray: And for plasma?

Vallance-Owen Yes. The other point is that we take good care to ensure that the animals are of the same weight and sex for any one experiment.

methods of assay rather than in obtaining results which are difficult to interpret. *We have studied insulin activity in the period before the*

response. There was no significant deviation from linearity with this

are allowed to use any transformation to give a straight line.

Diczfalusy: I have seen a Japanese report on male sex hormone assays

Astwood I wonder though, if you plotted in a more conventional manner whether you couldn't pick a range which might give parallel responses

experiments that λ was equal to 0 354 which, to be absolutely on a cube root basis, would have to be 0 333, but from this figure we feel that the transformation we use is reasonable.

FACTORS INFLUENCING THE LEVEL OF ACTH IN THE BLOOD*

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INVESTIGATION of the factors which influence the level of ACTH in the blood must include the determination of (1) the rate of excretion, (2) the rate of degradation, and (3) the rate of elaboration of the hormone. No detectable quantities of ACTH activity appear in the urine of rat (Richards and Sayers, 1951) or of man (Sayers *et al.*, 1949) following the intravenous administration of the hormone. A degradation product of the hormone may be excreted by the kidney, but no significant amount of biological activity is lost by this route. The very short half-life of endogenous ACTH, namely one minute (Sydnor and Sayers, 1953), indicates that the hormone is rapidly degraded in the body. Five minutes after the intravenous injection of ACTH no detectable activity was found in the liver. Less than 1/500th of the injected dose could be recovered from the adrenals. On the other hand, the kidneys accumulated one-fifth of the material and it would appear that renal tissue has a relatively high affinity for ACTH.

Selective accumulation in kidney and degradation at unknown sites result in a very brief sojourn of ACTH in the blood. Unfortunately, we have no information as to the possible effects of stressful stimuli on the factors which determine the rate of disappearance of ACTH from the blood. Our attention has been directed towards the pituitary for the elucidation of

[illegible] from the American Cancer Society on recommendation of [illegible]

the mechanisms whereby stressful stimuli increase the concentration of ACTH in the blood. The present communication is concerned in particular with neural mechanisms for regulation of release of ACTH from the adenohypophysis.

A relatively simple technique has been employed for the determination of the concentration of ACTH in the blood (Fig. 1). The left adrenal is removed from a hypophysectomized test rat for ascorbic acid analysis. Four ml. blood are

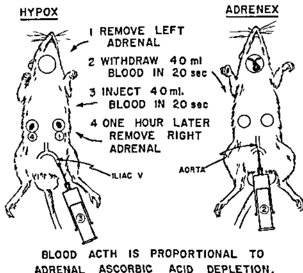


FIG 1. Method for the determination of the concentration of ACTH in blood.

drawn from the abdominal aorta of a donor rat in approximately 20 seconds and rapidly transferred to the hypophysectomized recipient One hour later the right adrenal is removed from the recipient rat for ascorbic acid analysis. The quantity of ACTH in the 4 ml. blood from the donor rat is proportional to the depletion of adrenal ascorbic acid.

Ether anaesthesia induces a rapid and marked increase in blood ACTH in adrenalectomized rats. The non-stressed or

FACTORS INFLUENCING THE LEVEL OF ACTH IN THE BLOOD*

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Investigation of the factors which influence the level of ACTH in the blood must include the determination of (1) the rate of excretion, (2) the rate of degradation, and (3) the rate of elaboration of the hormone. No detectable quantities of ACTH activity appear in the urine of rat (Richards and Sayers, 1951) or of man (Sayers *et al.*, 1949) following the intravenous administration of the hormone. A degradation product of the hormone may be excreted by the kidney, but no significant amount of biological activity is lost by this route. The very short half-life of endogenous ACTH, namely one minute (Sydnor and Sayers, 1953), indicates that the hormone is rapidly degraded in the body. Five minutes after the intravenous injection of ACTH no detectable activity was found in the liver. Less than 1/500th of the injected dose could be recovered from the adrenals. On the other hand, the kidneys accumulated one-fifth of the material and it would appear that renal tissue has a relatively high affinity for ACTH.

Selective accumulation in kidney and degradation at unknown sites result in a very brief sojourn of ACTH in the blood. Unfortunately, we have no information as to the possible effects of stressful stimuli on the factors which determine the rate of disappearance of ACTH from the blood. Our attention has been directed towards the pituitary for the elucidation of

* Aided by grants from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

† Editors' Note: As Prof. Sayers was unable to attend the meeting, his paper was presented for him by Dr. G. L. Farrell.

In contrast to ether, pentobarbital (4 mg./100 g. body weight) did not elevate blood ACTH. Pentobarbital may actually reduce the "resting" level of hormone in the blood.

Pitressin was injected intravenously at a constant rate (0·2 u./min.) into adrenalectomized rats under pentobarbital anaesthesia. Blood ACTH was markedly elevated at 5 and at 15 minutes, but approached "resting" levels at 30 minutes of continuous injection of Pitressin (Table III). The pituitary

Table III

BLOOD ACTH AFTER PITRESSIN (0 2 U./MIN., I V) IN ANAESTHETIZED (SODIUM PENTOBARBITAL) ADRENALECTOMIZED RATS

Treatment	*Time after beginning of injection*	*No rats*	*Ascorbic acid depletion induced by 4 ml. blood*
	min		
Anaesthesia only		7	12 ± 7*
Pitressin	5	8	134 ± 9
Pitressin	15	8	112 ± 14
Pitressin	30	8	58 ± 9

* Mean ± S E

response to Pitressin as well as to ether continuously applied is characterized by an "excitatory" and a "regressive" phase.

Pitressin induced a significant increase in blood ACTH in intact rats, but the rise was much less marked than in adrenalectomized rats.

Section through the brain stem at the upper level of pons abolishes the excitatory phase of the pituitary response to ether and to Pitressin (Table IV). Sham decerebrate animals exhibited a normal "resting" level of blood ACTH, and they displayed the usual marked elevation in hormone titre when exposed to ether or when injected with Pitressin. The "resting" level of ACTH in the decerebrate animal 70 to 200 minutes after section of the brain stem was not significantly different from that in sham-decerebrate rats, and this level was not appreciably increased by ether or by Pitressin.

resting level of ACTH in the blood (0 min. in Table I) was obtained as follows. The rat was handled gently and a neck clamp applied which stopped the flow of blood from the head

Table I

BLOOD ACTH IN ADRENALECTOMIZED RATS DURING ETHER ANAESTHESIA

Duration of ether anaesthesia min.	*No. rats*	*Ascorbic acid depletion induced by 4 ml blood*
0	9	42 ± 9*
2	21	109 ± 6
5	10	117 ± 8
15	8	63 ± 12
30	47	41 ± 6
60	8	12 ± 9

* Mean ± S E

to the trunk. This precluded a contribution by the adenohypophysis to the ACTH in the trunk during the collection of the sample from the abdominal aorta. At 2 and 5 minutes of ether anaesthesia blood ACTH was markedly elevated. After 15 minutes the level had declined, and after 30 minutes of ether anaesthesia the concentration of ACTH in the blood was the same as the pre-stress value.

ACTH was not detectable in the blood of the intact rat, either non-stressed or anaesthetized with ether (Table II).

Table II

BLOOD ACTH IN INTACT RATS DURING ETHER ANAESTHESIA

Duration of ether anaesthesia min.	*No. rats*	*Ascorbic acid depletion induced by 4 ml. blood*
0	11	6 ± 9*
2	13	−2 ± 9
5	10	−11 ± 8
15	7	4 ± 9
30	14	−18 ± 7

* Mean ± S.E

However, in an earlier study (Sydnor and Sayers, 1954) in which pooled samples of blood were processed by the oxycellulose technique, a small but significant increase in the level of blood ACTH was demonstrated to occur in intact rats under ether anaesthesia for 5 minutes.

were dealing with a hypothalamic neurohumor which induces ACTH discharge. For this reason the following studies were carried out.

The hypothalamic tissue was excised from calf brains at the slaughterhouse. The tissue included median eminence and ventral hypothalamus as far caudal as the mammillary body and was cut at a depth of about 5 mm. The tissue was frozen

Table V

BLOOD ACTH AFTER EPINEPHRINE AND HYPOTHALAMIC EXTRACTS IN DECEREBRATE ADRENALECTOMIZED RATS

	Ascorbic acid depletion induced by 4 ml. blood	
	Adrenalectomized Sham Decerebrate	*Adrenalectomized Decerebrate*
5 ml. 0 1N-HCl in 0 9 per cent NaCl	—	29 ± 16 (10)
Ether—2 min.	116 ± 6 (9)	29 ± 11 (13)
Pitressin—5 u.	148 ± 13 (13)	45 ± 10 (11)
Epinephrine—10 µg.	—	105 ± 10 (8)
Norepinephrine—10 µg.	—	82 ± 15 (7)
Cerebral cortex—0·1N-HCl extract	105 ± 7 (7)	56 ± 6 (6)
ME No.1. 0 1N-HCl extract of ventral hypothalamus	—	158 ± 13 (9)

in dry ice and brought to the laboratory where it was extracted with 0·1N-HCl in a Waring blendor and centrifuged. The supernatant was highly toxic. Heating for 10 minutes at 100°C before centrifugation resulted in an extract which was well tolerated by the animals. Two ml. extract equivalent to one ventral hypothalamus was injected into a saphenous vein over 5 minutes A neck clamp was applied at the end of the injection period and 4 ml. blood was drawn from the abdominal aorta for ACTH analysis.

A small quantity of ACTH activity was present in extracts of ventral hypothalamus. In order to establish the presence

Table IV

BLOOD ACTH AFTER ETHER AND PITRESSIN IN DECEREBRATE ADRENALECTOMIZED RATS

Treatment	*Period of exposure or injection*	*No. rats*	*Ascorbic acid depletion induced by 4 ml. blood*
	min.		
Sham decerebration		6	24 ± 9*
Sham decerebration—0 9 per cent NaCl, i.p	5	4	14 ± 9
Sham decerebration—ether	2	9	116 ± 6
Sham decerebration—5 u. Pitressin, i.p.	5	13	148 ± 13
Decerebration		4	11 ± 20
Decerebration—5 ml. 0 1N-HCl in 5·9 per cent NaCl, i.v.	5	10	29 ± 16
Decerebration—ether	2	13	29 ± 11
Decerebration—5 u. Pitressin, i.p.	5	11	45 ± 10

* Mean ± S E.

These experiments indicate that neither ether nor Pitressin acts directly on the hypothalamus or the pituitary. The receptors and the neural pathways which subserve these noxious agents in their stimulatory action on the pituitary are unknown. Experimental studies of Porter (1952) and of Wilson and co-workers (1956) support the thesis that deafferentation of the hypothalamus abolishes the response of the pituitary to certain noxious stimuli. However, certain agents act to induce ACTH discharge at a level in the brain rostral to the pons. Epinephrine and extracts of the ventral hypothalamus increase blood ACTH in the decerebrate rat (section at upper level of pons) (Table V). Norepinephrine may be less active than epinephrine, and extracts of cerebral cortex (weight of tissue extracted equivalent to that of ventral hypothalamus) had little if any activity.

The finding that extracts of ventral hypothalamus induced ACTH discharge in the decerebrate rat suggested that we

extracts (ME No. 5 and ME No. 6) were devoid of activity. The active factor may not be extracted at neutral pH or it may be destroyed, for example, by proteolysis. Oxycellulose was added to an acetic acid extract and an active fraction was eluted with 0·1N-HCl (ME No. 7). Activity remained after lyophilization (ME No. 7a). The results suggest that we are dealing with a basic polypeptide. Obviously, no definitive statement can be made about the chemical nature of the factor at this stage of development of the experimental work.

Now we would like to return to a consideration of the "regressive" phase of the response of the pituitary to Pitressin or ether. The regressive phase of the response of the pituitary to ether or to Pitressin is not due to cardiovascular collapse or to exhaustion of pituitary stores of ACTH. The content of ACTH in the pituitaries of rats during the regressive phase is, if anything, slightly higher than that of controls, as determined by direct assay of the glands. Further, the blood pressure and rectal temperature are normal at this time. The phenomenon may be explained on the basis of tolerance of the animal to the noxious agents. As the animal develops resistance the excitation of the pituitary lessens. Alternatively, an element of the neural mechanism concerned with enhancement of pituitary ACTH activity may exhibit adaptation.

Experimental work in other laboratories has uncovered a biphasic response to noxious agents and extremes of the environment For example, Briggs and Munson (1955) have demonstrated that morphine initially induces excitation of the adenohypophysis, followed by a period in which the drug may actually block the effects of other noxious stimuli. Exposure of dogs to an extremely cold environment (— 50° or — 75°C) was associated with an initial rise followed by a decrease in the adrenal output of 17-hydroxycorticosteroids (Egdahl and Richards, 1956).

If adaptation occurs, it does not involve that segment of the neural mechanism which represents a final common path (pons to hypothalamus to adenohypophysis?) for all noxious

of a factor in the extract which induced release of ACTH from the pituitary of the decerebrate rat, comparison was made of the ACTH content of blood from adrenalectomized decerebrate rats with blood from adrenalectomized hypophysectomized rats injected in identical fashion with extract of the ventral hypothalamus. Such a comparison is presented in Table VI. Three hydrochloric acid extracts (ME No. 1, ME

Table VI

BLOOD ACTH AFTER HYPOTHALAMIC EXTRACTS IN DECEREBRATE ADRENALECTOMIZED AND IN ADRENALECTOMIZED HYPOPHYSECTOMIZED RATS

	Ascorbic acid depletion induced by 4 ml blood	
	Adrenalectomized decerebrate	*Adrenalectomized hypophysectomized*
ME No 1. 0·1N-HCl extract of ventral hypothalamus	158 ± 13 (9)	62 ± 16 (4)
ME No. 2. Same as No 1	172 (133, 206, 178)	83 ± 20 (6)
ME No. 3. Same as No 1	144 ± 12 (5)	2 (−22, 4, 23)
ME No 4 0 2N acetic acid extract of ventral hypothalamus	126 ± 15 (7)	34 ± 9 (6)
ME No. 5. Neutral saline extract of ventral hypothalamus, room temperature	35 ± 5 (6)	—
ME No. 6. Neutral saline extract of ventral hypothalamus, 4°C	48 ± 8 (6)	—
ME No. 7. Oxycellulose eluate acetic acid extract of ventral hypothalamus	109 ± 15 (7)	29 ± 21 (5)
ME No. 7a. ME No 7 lyophilized	111 ± 25 (4)	—

No. 2, and ME No. 3) induced a significantly higher level of ACTH in the blood of adrenalectomized decerebrate rats than in the blood of adrenalectomized hypophysectomized rats, and we interpret this to mean that these extracts contained a substance which excited the pituitary to release ACTH. An acetic acid extract (ME No. 4) was also active. Neutral

Concluding Remar

Noxious stimuli increase the concentr
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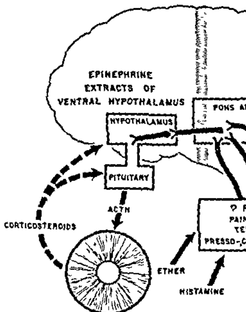


FIG. 2. Mechanisms concerned with the regulat
the pituitary.

the corticosteroids on ACTH release
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system operates to regulate ACTH out

stimuli. Adaptation *must occur in elements of separate paths* which subserve various categories of noxious stimuli and which are located distal to the final common path. Pitressin injected between the 30th and 35th minute of ether anaesthesia will induce a marked increase in blood ACTH (Table VII).

Table VII

BLOOD ACTH IN ADRENALECTOMIZED RATS SUBJECTED TO TWO NOXIOUS STIMULI APPLIED IN SEQUENCE

Treatment	*No. rats*	*Ascorbic acid depletion induced by 4 ml. blood*
Ether for 30 min.	47	41 ± 6
Pitressin i.p.—wait 5 min.	13	122 ± 15
Ether for 35 min. Pitressin i.v. injected from 30th to 35th min.	15	138 ± 11
Pitressin i.p.—wait 30 min	8	6 ± 2
Ether for 2 min.	21	109 ± 6
Pitressin i.p.—wait 30 min. —ether for 2 min.	9	116 ± 18
Histamine i.p.—wait 5 min.	9	133 ± 16
Ether for 35 min. Histamine i.v injected from 30th to 35th min.	12	54 ± 14

Adaptation to ether obviously does not interfere with the response to Pitressin. The two noxious stimuli may be presented in reverse sequence. Ether for two minutes starting 30 minutes after the intraperitoneal injection of 5 u. Pitressin induced a marked increase in blood ACTH. Pitressin does not block the response to ether.

However, ether does block the response to histamine. Histamine administered intravenously between the 30th and the 35th minute of ether anaesthesia did not increase blood ACTH. The results suggest that ether and histamine utilize in common at least one segment of a neural pathway distal to the final common path.

is required before we can present an adequate explanation for the nature of the blocking effect of one noxious stimulus on another.

REFERENCES

BRIGGS, F. N., and MUNSON, P L (1955). *Endocrinology*, 57, 205.
EGDAHL, R. H., and RICHARDS, J. B. (1956). *Amer. J Physiol.*, 185, 239.
PORTER, R. W. (1952). *Amer. J. Physiol.*, 169, 629.
RICHARDS, J. B., and SAYERS, G. (1951) *Proc. Soc. exp. Biol., N.Y.*, 77, 87.
SAYERS, G., BURNS, T. W., TYLER, F. H, JAGER, B. V, SCHWARTZ, T. B., SMITH, E. L., SAMUELS, L. T., and DAVENPORT, H. W. (1949) *J clin. Endocrin.*, 9, 593.
SYDNOR, K. L., and SAYERS, G (1953). *Proc Soc exp. Biol., N.Y*, 83, 729.
SYDNOR, K. L., and SAYERS, G. (1954). *Endocrinology*, 55, 621
WILSON, H, ANDERSON, E., SPENCE, W. T., and RIOCH, D. McK. (1956). *J. clin. Endocrin.*, 16, 943.

[Discussion of this paper was postponed until after the paper by Prof. Prunty.—ED.]

The pituitary response to certain noxious stimuli is biphasic. An initial "excitatory" phase is associated with a rapid and marked elevation of blood ACTH, and is followed by a "regressive" phase during which the level of ACTH in the blood returns to the pre-stimulus level despite continuous action of the noxious agent.

The "excitatory" phase of the response to ether and to Pitressin can be abolished by high pontine section. Vasopressin (antidiuretic hormone) or a contaminant of vasopressin in commercial Pitressin does not act directly on the hypothalamus or the adenohypophysis to induce ACTH discharge. The evidence presented here does not support the thesis that vasopressin is the neurohumor elaborated by the median eminence to regulate pituitary ACTH activity.

Epinephrine and acid extracts of the ventral hypothalamus elevate blood ACTH in adrenalectomized decerebrate rats. These agents appear to act rostral to the pons. We must await the development of a more specific assay object before the significance of these findings to pituitary control can be established.

The prompt discharge of ACTH in response to a noxious stimulus may represent one manifestation of a generalized reaction of the hypothalamus. Supraoptic nuclei, autonomic centres, and centres concerned with pituitary activation (ACTH) may all join in an indiscriminate response to a noxious agent.

The "regressive" phase of the response of the pituitary to noxious stimuli may be a consequence of the acquisition of tolerance on the part of the animal or it may represent an adaptation of a segment of certain neural pathways concerned with excitation of the adenohypophysis. We have speculated on the blocking action exhibited by certain noxious agents which are themselves excitatory in the early period of their action on the pituitary. It has been suggested that a noxious agent will block another if they utilize a common segment of a neural pathway which undergoes adaption. It is obvious that more detailed information about the neural pathways

period of partial depletion during which detectable amounts of ascorbic acid are present in the adrenal (Prunty and Clayton, 1955) and a "terminal period" which includes the few days immediately prior to death of the animal, when the adrenal is completely depleted of the vitamin. This period is marked by the appearance of haemorrhages in the gums and intestine. Death can then be predicted to occur within two days. In the terminal phase the adrenal cortex is hypertrophied, but this can be reversed by administration of cortisone or ascorbic acid. There is also a rise in urinary ketosteroids and ketogenic steroids, accompanied by a big increase in plasma cortisol.

Various observers have reported a fall in urinary ketosteroids in scurvy (see Bacchus, 1954), and we have observed it in patients (Prunty and Clayton, 1955). It can readily be observed during scurvy which is allowed to develop more slowly by administering 2 mg. ascorbic acid every fourth day to guinea pigs (Clayton, Mills and Prunty, 1954). In most acute experiments using complete deprivation of ascorbic acid the fall can be picked up in the early period, but occasionally this may not occur or be missed (Fig. 2). It is therefore important in assessing the adrenal response to take careful account of the severity of the condition at the time of observation. Others have found increased corticoids in blood and urine in severe scurvy (Done *et al.*, 1953; Nadel and Burstein, 1956). The latter authors also found evidence in the adrenal of unusual cortisol synthesis from acetate. That much free steroid, largely cortisol, is excreted in normal guinea pig urine in response to corticotrophin has been found by us, this fraction representing 70 per cent of the ketogenic steroids. Burstein and Dorfman (1954) found a preponderance of cortisol and 6β-hydroxycortisol in the urine of these animals. We, therefore, believe that the terminal scorbutic period is one in which considerable activation of the pituitary-adrenal system occurs. On the other hand, Bacchus (1954) has advanced the view that there is a failure of the system under these conditions, and the amount of hormone secreted is

EXPERIMENTS ON THE LEVEL OF BLOOD CORTICOTROPHIN WITH PARTICULAR REFERENCE TO SCURVY

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PREVIOUS work in our laboratory has indicated that experimental scurvy in the guinea pig is associated with greatly

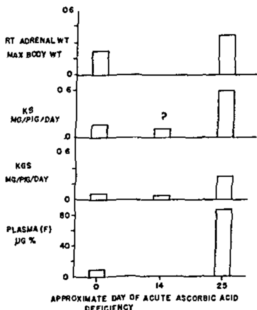


FIG. 1. Changes in adrenal weight, ketosteroid and ketogenic steroid excretion and plasma cortisol in acute scurvy in guinea pigs.

increased adrenal cortical activity. In Fig. 1, the evolution of the scorbutic state has been divided into two phases, an early

of the blood corticotrophin in these scorbutic animals. In the experimental procedure guinea pig serum has been used (Armitage, Clayton and Hammant, 1957) and the assays were carried out in rats with slight modifications of the Sayers method (Clayton and Prunty, 1951). In addition to these, animals weighing up to 160 g. have been used, and those animals rejected in the assay in which the difference in weight

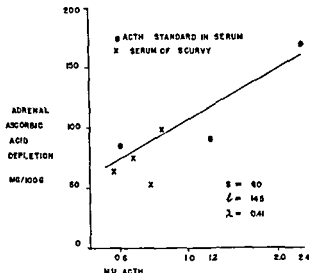


FIG. 3. Response given by guinea pig serum from scorbutic animals in the Sayers test.

between the two adrenal glands exceeded 2 mg., since this is found to contribute to the occurrence of aberrant results. Fig. 3 shows the regression calculated to fit observations on laboratory standard corticotrophin in normal guinea pig serum which itself contains no activity, and observations on the serum of male guinea pigs allowed to develop scurvy for 23 days. The guinea pigs weighed 350 to 450 g. and were fed on the diet of Harris and Ray (1932). Animals of this particular size normally die at the 25th or 26th day.

The scorbutic serum is estimated to possess 40 i.mu./100 ml.

reduced. The question has also been raised concerning the specificity of the response in scurvy. That the response is not due merely to the severe appetite loss of the animals is shown in Fig. 2, where comparison of the steroid excretion with that of pair-fed controls is made. In the pair-fed animals the ketogenic steroids did not rise and the increase of ketosteroids was smaller. It may further be argued that the pre-mortal condition of the scorbutic animals is alone sufficient to cause the

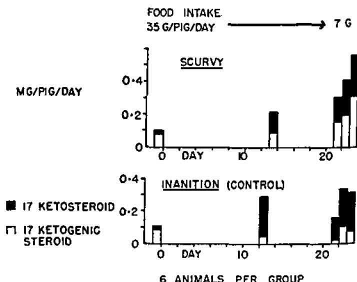


FIG. 2. Excretion of ketosteroids and ketogenic steroids in the urine of guinea pigs with acute scurvy and of pair-fed controls.

observed rise in hormone level. Sandberg and co-workers (1956) have shown that in dying patients there is a sharp pre-terminal rise in plasma 17-hydroxycorticoids, but this was largely due to failure of metabolism of free cortisol which in turn is less readily cleared by the human kidney. It would therefore seem that the conditions differ from those described here where there is a rise of both blood level and rate of excretion.

For these reasons it has become important to study the level

steroids were given in two doses of 10 mg., 24 hours and 1·5 hours before assay of corticotrophin. In addition to cortisol,

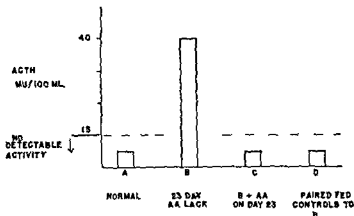


FIG. 4. Absence of detectable corticotrophin in serum of pair-fed controls to scorbutic animals and of serum of the latter animals given ascorbic acid (see text).

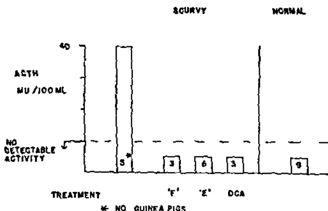


FIG 5 Suppression of corticotrophin with corticoids.

cortisone and desoxycorticosterone acetate were both effective. Progesterone, oestradiol benzoate and testosterone did not

serum (fiducial limits 0·84–82). The largest dose of serum which it is practicable to administer to rats is 2 ml., and hence the points fall at the lower end of the standard curve.

In considering the validity of the data on single point observations it is calculated that if $P = 0{\cdot}05$, a single dose assay with 4 rats will detect 0·6 mu. corticotrophin, with an ascorbic acid depletion of 60 mg./100 g. adrenal tissue. This is half the level of sensitivity found by Sayers and his group using a similar number of assay animals, and we are therefore certain of being unable to detect levels below 0·3 mu., corresponding to a minimal serum level of 15 mu./100 ml.

To substantiate the validity of this contention the following may be cited. A patient with rheumatoid arthritis received during 24 hours an intravenous infusion of 100 i.u. corticotrophin. Allowing the liberal half-life of five minutes (Sayers, 1955; Sydnor and Sayers, 1953) this would give a plasma level of 14 mu./100 ml. Two ml. of the plasma at the termination of the infusion gave an ascorbic acid depletion of 70 ± 15 mg./100 g, suggesting a plasma concentration of about 30 mu./100 ml. On the other hand, we have been unable to detect corticotrophin in the plasma of rats adrenalectomized 7 to 10 days previously and maintained on glucose-saline. Corticotrophin levels about 3 mu./100 ml. rat blood have been reported after adrenalectomy in rats (Sydnor and Sayers, 1954).

In the scorbutic guinea pig the high level of corticortophin is depressed to undetectable levels by the administration of "saturating" amounts of ascorbic acid on the 23rd day (1 g. orally, 1 g. sodium ascorbate intraperitoneally), given 1·5 hours before killing (Fig. 4). Pair-fed controls to the scorbutic animals show no activity. We have previously found that 20 mg. ascorbic acid given on the 25th day of vitamin deple- [illegible] also reduce [illegible] w detect- [illegible] d in this experiment was 10 mg. subcutaneously 24 hours before killing, [illegible] 1·5 hours before killing. The other

To test the effect of ascorbic acid given in excess of normal amounts to animals which had not been depleted of the

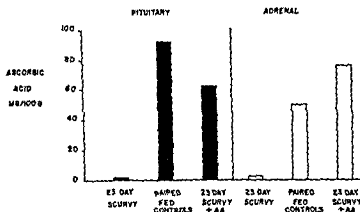


FIG 8. Pituitary and adrenal content of ascorbic acid

vitamin preliminary observations have been made (Fig. 9). Guinea pigs were adrenalectomized and maintained on 12·5

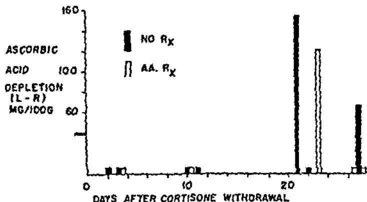


FIG 9. Failure of ascorbic acid to suppress the appearance of corticotrophin in the serum of adrenalectomized guinea pigs

mg. of cortisone daily for the first two weeks after the operation, and 12·5 mg. 4 times weekly if they have been adrenalectomized for a longer period. Two ml. guinea pig serum

reduce corticotrophin below detectable amounts (Fig. 6). In the scorbutic guinea pigs the pituitary glands contain corticotrophin in amounts comparable with those of normal animals

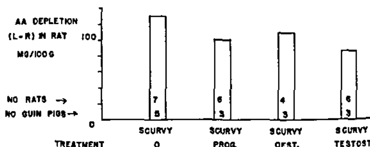


FIG. 6. Failure to suppress corticotrophin with oestradiol, progesterone and testosterone.

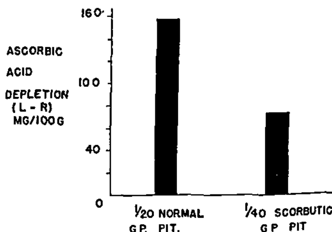


FIG. 7. Similar pituitary corticotrophin content of normal and scorbutic guinea pig pituitaries.

(Fig. 7), but the concentration of ascorbic acid has decreased to undetectable amounts, as it has in the adrenal (Fig. 8).

Heiffer and Altszuler (1952) noted that ascorbic acid blocked the eosinopenic response to adrenaline; Bacchus, Altszuler and Heiffer (1952) found a synergism between ascorbic acid and corticoid effects on peripheral blood cells, and gluconeogenesis. Most important perhaps are the many experiments illustrating a depressive effect of ascorbic acid on the mortality of cold stress in both animals requiring dietary ascorbic acid and in those synthesizing their own (Dugal and Therien, 1949; Booker *et al.*, 1955). Indeed the presence of the adrenal seems to be necessary for this response, and would be compatible with a synergistic effect of the vitamin and cortical hormone on pituitary secretion. Such an argument is favoured by the preliminary results, here described, on the lack of effects of ascorbic acid on the corticotrophin in adrenalectomized guinea pigs. It is noteworthy that in these animals some time must elapse after cortisone withdrawal before large amounts of corticotrophin appear in the blood. This is reminiscent of the difficulty of detecting plasma corticotrophin in patients with the adrenogenital syndrome after cessation of cortisone treatment (Sydnor *et al.*, 1953).

There still, however, remains the problem of the non-specific pre-mortal condition of our animals in severe scurvy. Reasons have already been mentioned for thinking that the pre-mortal conditions observed in man by Sandberg and co-workers (1956) produce effects of a different character from those in the scorbutic guinea pigs. We have attempted to produce a measurable increase of corticotrophin by the administration of carbon tetrachloride over a 6-day period to 6 intact male guinea pigs. These animals were not deprived of ascorbic acid, and although moribund at the time of killing the serum corticotrophin was undetectable, the ascorbic acid depletion in 5 test rats being −82 mg./100 g. In spite of these difficulties, Nadel and Burstein (1956) have been able to produce significantly increased secretion of cortical and related steroids in guinea pigs with acute experimental leukaemia. It would therefore be safest to assume for the present that the overall effects seen in acute scurvy could be accounted

were injected into each of at least 4 test rats. It seems that after cortisone withdrawal several days must elapse before detectable quantities of corticotrophin appear in the serum. Corticotrophin was still detectable at 23 days after withdrawal of cortisone when 2 g. ascorbic acid was given orally 1·5 hours before killing.

Discussion

According to the computation of Sayers (1955) a level of 40 mu./100 ml. for corticotrophin in the serum of scorbutic guinea pigs would appear to be a maximal one, and expected to produce intense stimulation of the adrenal cortex. The weight of evidence that the cortex is so stimulated has already been considered above, and it must therefore be that scurvy is acting as a severe stress in which there is a balanced elevation of plasma corticoid and corticotrophin, the major stimulus to the pituitary arriving perhaps by the neurotropic route (Sayers, 1950). It would be expected that further elevation of the peripheral corticoid by the administration of suitable substances, of which cortisol, cortisone and desoxycorticosterone were given in large doses, would depress the corticotrophin secretion. The point of special interest is whether ascorbic acid deprivation is in any way specific in the observed responses, other than merely responsible for the production of a severe stress Experiments using pair-feeding alone do not settle this problem, for after 20 days animals so fed are in a better condition than the corresponding scurvy animals. Evidence exists suggesting some specific effects of ascorbic acid on the pituitary adrenal system. Stepto and collaborators (1952) considered that a rise in the guinea pig pituitary content of corticotrophin occurred as a result of ascorbic acid depletion. This rise seems to be too small to be detectable in our experiments. The short time of 1·5 hours elapsing between the administration of ascorbic acid to scorbutic animals and the depression of the corticotrophin in the serum might suggest a direct action of the ascorbic acid rather than the complete abolition of the "stresses" of scurvy. Bacchus,

DISCUSSION

Harris: I have many questions I should like to ask Dr. Farrell, after

of adrenal steroids in inhibiting ACTH secretion were at a reticular system level, then loss of consciousness might be expected to follow. I wonder if you have any views on a possible relationship between your results and the brain stem reticular formation

Farrell: I know that Sayers has entertained the possibility that the reticular system may play some part in this phenomenon. Spinal section at the level of, I think, C_5 to C_7 does not abolish the response to ether. One may speculate that the stimulating agent acts directly on the reticular formation On the other hand, section of the 5th nerve which was a consequence of the brain stem section results in the interruption of many afferent pathways from the oral mucosa Failure of ether to excite the pituitary may have simply been due to blocking the irritant

effect of hypothalamic lesions on the action of the extract, or have you tried direct micro-injection of the extract into the pituitary gland to see if that would elicit a response?

Farrell. No, the group is very actively attempting to establish specific hypothalamic lesions, which would, for example, prevent the effect of epinephrine.

Harris: We were unable to block the action of adrenaline in rabbits by pituitary stalk section (Fortier, C , Harris, G. W., and McDonald, I. R (1957). *J. Physiol.*, in press), but of course there is a discrepancy in the results recorded in the literature on this point. There are two groups; those workers who find the action of adrenaline is blocked by median eminence lesions, and those who find it is not blocked by pituitary stalk section or pituitary transplantation. One would expect the

Farrell· Yes.

Harris· Have you tried extracting the posterior pituitary gland as differentiated from the median eminence to see if the same method of extraction gives an active extract?

Farrell· The neurohypophysis does indeed yield an active extract when assayed in the animal with intact brain stem. But as far as I know

for by the acute stress invoked. Our experiments in the main have not enhanced the evidence for a direct effect of ascorbic acid on the pituitary-adrenal system, but have given additional support to the view that both the pituitary and adrenal cortex are capable of greatly increased activity in the absence of this mystifying substance.

Whilst the use of untreated serum with our colony of rats is sufficient to show the presence of corticotrophin under extreme conditions, its lack of sensitivity indicates the importance of concentrating the hormone before assay as suggested by Sydnor and Sayers (1952).

REFERENCES

ARMITAGE, P., CLAYTON, B. E., and HAMMANT, J. E. (1957). *J. Endocrin.* In press.

BACCHUS, H. (1954). *In* Fourth Annual Report on Stress, Selye, H., and Heuser, G., p 127. Montreal: Acta Inc.

BACCHUS, H., ALTSZULER, N., and HEIFFER, M. H (1952). *Proc. Soc. exp. Biol., N.Y.*, 80, 88.

BACCHUS, H, HEIFFER, M H., and ALTSZULER, N. (1952) *Endocrinology*, 51, 94.

BOOKER, W. M., DA COSTA, F. M., TURLMAN, R. J., FROIX, C., and JONES, W. (1955) *Endocrinology*, 56, 413

BURSTEIN, S., and DORFMAN, R I. (1954) *J. biol. Chem.*, 206, 607.

CLAYTON, B E., MILLS, I. H., and PRUNTY, F. T. G. (1954). *J. Endocrin*, 11, vi

CLAYTON, B. E., and PRUNTY, F. T. G. (1951). *Analyst*, 76, 474.

DONE, A. K., ELY, R. S., HEISELT, L. R., and KELLEY, V. C. (1953). *Proc. Soc. exp. Biol, N Y.*, 83, 722.

DUGAL, L. P., and THERIEN, M. (1949). *Endocrinology*, 44, 420.

HARRIS, L. J., and RAY, S N (1932). *Biochem. J.*, 26, 2067.

NADEL, E. M., and BURSTEIN, S. (1956). *J. nat. Cancer Inst*, 17, 213

PRUNTY, F. T. G, and CLAYTON, B. E. (1955). Ciba Foundation Colloquia on Endocrinology, 8, 324. London: Churchill.

SANDBERG, A. A., EIK-NES, K, MIGEON, C. J., and SAMUELS, L. T. (1956). *J. clin. Endocrin*, 16, 1001.

SAYERS, G. (1950). *Physiol. Rev*, 30, 241.

SAYERS, G. (1955). *J. clin. Endocrin.*, 15, 754.

STEPTO, R. C., PIRANI, C. L., FISHER, S. D, and SUTHERLAND, K. (1952). *Fed. Proc.*, 11, 429.

SYDNOR, K. L., KELLEY, V. C., RAILE, R. B., ELY, R. S., and SAYERS, G. (1953). *Proc. Soc. exp. Biol., N.Y.*, 82, 695

N.Y. 79 432.

Astwood· This was a suspension, wasn't it? Probably a depôt effect.

Prunty· No, it was given orally.

Samuels Of course there will be a period of absorption.

Farrell· Unfortunately, we have no definitive experimental data on animals which would clarify the problem as to the duration of the inhibitory influence of corticosteroids on the functional activity of the adenohypophysis or the adrenal cortex.

Samuels. I think the figure of about 1 week is a good figure for return to normal output. This does not mean that completely normal *maximal* response has been restored. We found that normal maximal response could be restored in even less time if the ACTH infusion test is repeatedly run on the individual. But of course this involves repeated stimulation of the adrenal with ACTH, so that one could not say that this was the rate at which it would have returned if no external influence had been imposed upon the adrenals. There seems to be a considerable variation in the rate of return to normal levels After stopping corticosteroid treatment all normal subjects show a dip to quite low levels Some of these will return to normal levels in 48 hours, others in 72 hours, and others longer. This is now not testing whether they can respond maximally to stimulus.

Bush. Dr. Farrell, you found that ether would block your response to histamine and you therefore suggested that the peripheral receptors might be common to the two agents. Did you try the reverse experiment to see whether the effect of ether was blocked by prior administration of histamine?

Farrell No, I haven't tried that.

Bush Because there is quite a possibility that they may be acting by different peripheral receptors or even by different central effects, and until you have done the converse experiment you cannot really say that the peripheral receptors were the same in the two.

The other question I wanted to ask was: it appears a little strange to me, perhaps Dr Vogt can give much better figures on this question, but you found that your ether response was blocked by decerebration whereas your adrenal response was not The general experience in small animals of this type is that ether anaesthesia causes a very considerable release of adrenaline I wondered whether you considered the possible conflict between these results?

Farrell I can see that would add a complication. However, you will recall that experiments were conducted on adrenalectomized animals so that release of epinephrine from the adrenal medulla did not complicate the picture.

Bush I see, you have done this in adrenalectomized rats

Farrell Yes Ether induced ACTH release in the absence of the adrenal medulla

Bush· I think one very important area as far as man is concerned was

it has not been assayed in the decerebrate animal; as I see it the point

pheral levels these continued at high levels over the entire period of observation, some 4 hours, falling slowly towards the end. I think at that time we deduced that both in the human, where this picture in the peripheral circulation was observed and we could make some association

I think that Prof. Prunty's evidence indicates that reduced hepatic function could not be a major factor here because he has good evidence of the increase in ACTH, which I am sure also occurs in the terminal

quite low,
nd, if you
ery much

anaesthetize the animal

increased, something of the order of 10–15 mu./100 ml.

Prunty: Does anyone have any information as to how long cortisone or cortisol would suppress the secretion of ACTH? From this data it look as though it may go on for some time

Samuels: Yes.

pointed out, comes in here

Samuels: Yes, that is true. It could be explained on that basis. The later has no relationship with the type of anaesthetic while the early changes are related to the type of anaesthetic. Ether, of course, being quite definitely a stimulus.

Vogt: As far as this question of ether is concerned, it is practically answered by what Dr Farrell has said, the amount of sympathomimetic amines which would be released by ether in the adrenalectomized animal is not likely to be sufficient to produce any release of ACTH, so that in those animals the ether must act by some other mechanism, in the intact animal the picture is more complicated and the effect of released adrenaline cannot be neglected.

Dr Farrell, concerning the Pitressin, do you know which preparation was used and how pure it was? And whether du Vigneaud's pure Pitressin would give the same effect, or whether another polypeptide from the posterior lobe is responsible?

in the decerebrate lesions animal, one was epinephrine and the other was the extract. I wonder if by any chance what Dr Sayers may be

you give the epinephrine in the decerebrate preparation?

Farrell. Yes

Vogt Then I think it is too little for producing an effect I think it may be one of these polypeptides which is responsible.

Savard: Coming back to the point of the rise in plasma corticosteroids

left out of the diagram and that is the influence of higher centres. Our experience, measuring both blood steroids and the output of steroids in the urine, is entirely parallel with that of Dr. G. W. Thorn, in that emotional stimulation is the only mode that can be relied upon in man to produce a really considerable increase in plasma steroids or the output of urinary steroids. If one looks carefully through the literature of results that have been obtained in experimental animals (if one allows them an emotional life of their own) and in man undergoing physical stresses, one cannot exclude the effect of unpleasant emotions. We have failed to stimulate the normal adrenal cortex by cold exposure or exercise. Emotional tension, however, such as an interview for a job which meant considerable family upheaval, or sitting for examinations, *produced enormous increases in urinary steroid output.* The actual figures were in the middle range for cases of moderate to severe Cushing's syndrome in our experience.

I think this is a very striking feature of the adrenal cortex in man, and so far all those stimuli which we regard as classical stimuli of the pituitary-adrenal system, namely physical stresses of one kind or another, do not seem to stimulate adrenocortical secretion in man, whereas emotional stimuli do.

Farrell: Certainly, emotional stimuli are able to increase the activity of the pituitary-adrenal system. Wouldn't you think that it would be rather surprising if it was not true, since many of the vegetative functions are affected by emotions? For example, we know that functions such as respiration, blood pressure and heart-rate are affected by the emotions. These are controlled by the medulla, yet are obviously affected by phenomena arising high in the cortex. So it would not be

esponds in much

what you might

think would be the pathway by which this response is set off?

however vaguely, that what we call the higher centres are involved. But I don't know whether there have been studies in lobotomized or leucotomized patients.

Samuels: I don't know of any. I would say that, in those studies which were done on the surgical patients, the later rise which tended to

CORTICOSTEROID-RELEASING ACTIVITY IN BLOOD*

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Introduction

THE measurement of circulating levels of the protein and polypeptide hormones is beset with many pitfalls. In the absence of specific chemical procedures, only biological methods of assay are applicable to this problem. It would seem essential that the specificity of any bioassay procedure must be rigidly established before it can be employed in the detection and estimation of the minute amounts of hormonal material normally present in the body fluids. And yet, the fact that this condition has probably never been realized has not particularly discouraged such investigations.

In addition to problems of specificity, the possibility of "masking" or potentiation of hormonal activity must be evaluated seriously. This is particularly true when the concentration of a "trace" substance is being measured in a medium containing relatively enormous amounts of related substances; for example, protein hormones in the presence of blood proteins. Moreover, when purification or concentration methods are employed, the prospect that hormonal activity has been lost or altered must be considered. Finally, the recovery of exogenously-added (even homologous) hormone cannot *a priori* be considered a reliable indicator for the behaviour of the endogenous material.

* [illegible]

in the human, we have just begun a very short series on surgical patients, studying plasma corticosteroid levels at 2-hour intervals, and we have noted that there is the apprehension rise prior to any anaesthesia and of course prior to surgery, but the rise is extremely small, perhaps 3 µg. per cent. That would raise a normal value of 8 to about 12, or 12 to about 15 µg per 100 ml. However, the anaesthesia will raise the levels to about 25 to 30 µg. per 100 ml., and then immediately after surgery they will go up further to levels of about 35–38. Now whether we must look at different forms of emotion remains to be seen. We have seen one case of a patient injured in an automobile accident in which the blood sample was taken 2 hours after the accident and that showed us the highest values we have ever encountered, in excess of 40 µg. per 100 ml. In this instance the emotional effect may be considered to be absent.

may not always agree with measurements based upon corticosteroid release *in vivo* (cf. Liddle *et al.*, 1954) or *in vitro* (Birmingham *et al.*, 1956), there appears to be no justification at the present time for assuming that the ascorbic acid assay is at fault (cf. Śtack-Dunne and Young, 1954).

During the past few years, two additional sensitive procedures for the estimation of ACTH have made their appearance. Both methods depend upon the stimulation of corticosteroid release, but with this difference: the first procedure measures corticosteroid secretion into the adrenal vein in the hypophysectomized dog (Nelson and Hume, 1955), the second, corticosteroid secretion by rat adrenal tissue *in vitro* (cf. Saffran and Schally, 1955*a*). The *in vivo* technique has somewhat less sensitivity than the ascorbic acid assay (i.e., about 1·0 mu. ACTH) and yields values for circulating ACTH (Nelson and Hume, 1955) comparable to those obtained by the latter procedure (cf. Gemzell *et al*, 1951; Sydnor and Sayers, 1952, 1954). The *in vitro* assay *appears* to have a sensitivity comparable to that of the *in vivo* method. However, it has been reported (Saffran and Schally, 1955*b*; Birmingham *et al.*, 1956) to yield values for the concentration of ACTH in the rat pituitary far in excess of those found using the ascorbic acid assay (Burns *et al.*, 1949; Gemzell *et al.*, 1951; Sydnor and Sayers, 1954). The *in vitro* method has not previously been applied to the measurement of blood ACTH, nor has its specificity been thoroughly investigated. The present report includes such studies. It will be demonstrated below that the *in vitro* corticosteroid-release procedure for the assay of ACTH is highly non-specific, since proteins or protein-bound substances other than ACTH are capable of stimulating this release. In addition, evidence is presented that the rat adrenal gland *in vitro* may be markedly responsive to rat ACTH and much less responsive to ACTH from other sources.

Whenever possible, results obtained by the *in vitro* technique have been compared with those obtained in this or other laboratories employing the hypophysectomized rat for the assay of ACTH and measuring the adrenal ascorbic acid

Ideally, a bioassay technique should measure a direct effect of the hormone studied and, preferably, that effect which is presumed to be the primary action of the hormone. To date, it has been impossible to agree upon a primary action for any hormone and it is not always as clear as it should be what constitutes a "direct effect". To illustrate from the problem at hand: it may be argued that the stimulation of corticosteroid release by the adrenal gland reflects a direct action of ACTH, whereas adrenal ascorbic acid decline under these circumstances may be an indirect action (cf. Nelson and Hume, 1955; Birmingham *et al.*, 1956). However, these conclusions no longer seem secure in view of the recent observation that ACTH-stimulated secretion of ascorbic acid into the adrenal venous effluent of the rat precedes the release of corticosterone (Slusher and Roberts, 1956). Incidentally, the latter investigators demonstrated that the ascorbic acid lost from the rat adrenal during enhanced adrenocortical activity could be quantitatively recovered in the adrenal venous effluent.

A number of assay procedures have been developed for the estimation of ACTH activity. We shall not be concerned here with those methods which obviously measure secondary responses of the organism to elevated circulating corticosteroids. The limitations of these techniques (for example, thymus atrophy, eosinopenia, etc.) are too well known to require comment. The maintenance or repair of adrenal size in hypophysectomized animals can also be dismissed as inapplicable to the measurement of minute amounts of circulating ACTH, even if other considerations did not make these criteria unreliable (cf. Stack-Dunne and Young, 1954). The adrenal ascorbic acid response to ACTH, discovered and developed by Sayers and Long and their collaborators (cf. Long, 1946; Sayers and Sayers, 1948), has generally been considered the most reliable and sensitive assay procedure available when properly employed. A significant response can be elicited in the hypophysectomized rat 1 day after operation with 0·2 mu. ACTH (cf. Sayers, Sayers and Woodbury, 1948). Although results obtained by this procedure

fied ACTH on corticosteroid release by rat adrenal tissue *in vitro* yielded results largely confirmatory of those obtained by the McGill group and described briefly above. Some of these data are depicted in Fig. 1. Male Sprague-Dawley rats from our own breeding colony were used in the assays. The animals weighed 120 to 180 g. Fig. 1 and succeeding charts depict the release of "TOTAL" methylene chloride soluble material absorbing maximally at 240 mμ. and of substances possessing the α-ketol side-chain (giving a blue colour with blue tetrazolium) and exhibiting the chromatographic behaviour of "B" and "DOC" on filter paper. Attention has been drawn in the present investigations to the latter two substances, inasmuch as they accounted for approximately 80 to 90 per cent of the extra α-ketolic steroids released by the rat adrenal in response to the presence of ACTH and other stimulatory substances in the incubation medium (*vide infra*). Similarly, compounds "B" and "DOC" comprised at least 70 per cent of the extra ultraviolet-absorbing material released under such circumstances. Quotation marks have been retained around "B", "DOC" and "TOTAL" steroids, since rigid identification of these substances has not been attempted. In fact, the material labelled "B" may in reality be 11-epi-corticosterone (cf. Eisenstein, 1956). Other steroids were frequently noted in the incubation media, but almost always in smaller quantities than "B" and "DOC". Especially notable were substances having the chromatographic, spectrophotometric, and staining characteristics of Compounds F, E, A, and S.

All materials assayed for corticosteroid-releasing capacity *in vitro* were incubated in triplicate with the equivalent of two (quartered) rat adrenal glands. The pre-incubation and incubation periods were conducted precisely as described by Saffran and Schally (1955*a*). The methylene chloride extracts of the incubation media in the three similar flasks, after ultraviolet analysis, were routinely combined and subjected to quantitative chromatography. The extracts were evaporated to dryness under nitrogen, with a bath temperature below

response (Sayers, Sayers and Woodbury, 1948) or corticosteroid release into the adrenal venous effluent (Bush, 1952, 1953; Singer and Stack-Dunne, 1955, Beigelman *et al.*, 1956). Corticosteroid release in the hypophysectomized rat appeared to have approximately the same sensitivity to ACTH as the ascorbic acid response, but was not as readily quantitated (Slusher and Roberts, unpublished).

Brief Description of Technique

Corticosteroid release *in vitro* by rat adrenal tissue has been developed as a method of assay for ACTH by Saffran and his co-workers at McGill (cf. also, McKerns and Nordstrand, 1955). This procedure has been employed for the measurement of ACTH in the rat adenohypophysis (Birmingham *et al.*, 1956) and for the recognition of ACTH-releasing factors in neurohypophysial (Saffran, Schally and Benfey, 1955) and hypothalamic (Saffran and [illegible] tially, the [illegible] measure [illegible] in meth [illegible] maximally in the ultra-violet at [illegible] steroids possess [illegible] and non-[illegible] of thes [illegible] (Elliott and Schally, [illegible] increasing amounts of purified ACTH added to the incubation medium (c. 1·0 to 30 mu. U.S.P. Corticotrophin Reference Standard) the log. dose-response curve followed a straight line. The McGill workers, employing paper chromatographic separation of the steroids released to the medium, reported that α-ketolic steroids (giving a purple colour with the substituted tetrazolium reagent, M. and B. 1767) possessing the chromatographic mobilities of cortisol (F), corticosterone (B), and 11-desoxycorticosterone (DOC) normally comprised about 50 per cent of the total ultraviolet-absorbing material (Elliott and Schally, 1955). Under the influence of purified ACTH, mainly "B" secretion was increased.

Studies carried out in our laboratories on the effect of puri-

steroid followed a log. dose-response relationship in this range (cf. also, Saffran and Schally, 1955*a*; McKerns and Nordstrand, 1955). "DOC" release appeared to be unrelated to the amount of ACTH present, and represented a very minor

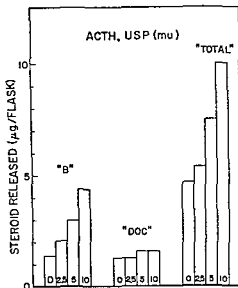


FIG. 1. The influence of purified corticotrophin (U S P Reference Standard) on the release of corticosteroids by rat adrenal tissue *in vitro*.

portion of the "TOTAL" figure. In the presence of cruder ACTH preparations (*vide infra*), "DOC" made up an appreciably larger proportion of the total steroids released.

Dilute acid (0·1N–HCl) extracts of the rat adenohypophysis (method of Sydnor and Sayers, 1954), after neutralization with bicarbonate buffer, were extremely active in promoting the

40°C, and then purified by partitioning between 70 per cent ethanol and petroleum ether. The ethanol solution was distilled to near dryness (under nitrogen) and the residue then taken up in ethyl acetate-methanol (1 : 1) for application by pipetting to filter paper (cf. Bush, 1952; Singer and Stack-Dunne 1955). Quantitation of the individual α-ketolic steroids, after chromatographic separation on Whatman No. 1 filter paper employing the toluene/methanol/water (4:3:1) system of Bush (1952), was accomplished by spraying the filter paper with blue tetrazolium, eluting each spot with 2 ml. ethyl acetate-methanol (7 : 3), and reading the optical density of the eluates at 565 mμ. in the spectrophotometer (cf. Touchston and Hsu, 1955). The reliability of these procedures, as applied to the measurement of corticosterone in rat adrenal venous effluent, has been described earlier (Beigelman *et al.*, 1956). Recovery of steroid standards added to the incubation media was close to 100 per cent. Standards of compounds B and DOC were run on each chromatogram at levels of 5 and 10 μg. and the colour values employed for the estimation of these steroids in the assay flasks. "TOTAL" steroid values were calculated from the ultraviolet absorption of the methylene chloride extracts (cf. Saffran and Schally, 1955*a*) compared with that of corticosterone standards in the same solvent. The latter calculations are subject to error as a result of quantitative differences in the ultraviolet absorption of B and other steroids present in the incubation media. In some instances, this error may have been appreciable (*c.* 10 to 15 per cent), since the major other steroid which appeared to be present (DOC) has spectrophotometric characteristics which are significantly different from B (Elliott *et al.*, 1954).

Corticosteroid-releasing Activity in Pituitary Extracts

The release of "B" *in vitro* was roughly proportional to the amount of purified ACTH added between doses of 2·5 and 10 mu. (Fig. 1). On the other hand, the release of "TOTAL"

amount of steroid released during a 2-hour period over and above the value obtained when adrenals were incubated in buffer without additives. In the presence of pituitary extracts (Fig. 2), large amounts of both "B" and "DOC" were released; the combined value for these two steroids approximated to the level of "TOTAL" steroid release. The release of "B" appeared to be a better measure of ACTH activity than the release of "DOC" or "TOTAL" steroid, inasmuch as the apparent loss of ACTH in sample II stored at room temperature (II O) was more obviously reflected in a decline in "B"-releasing activity than in "DOC"-releasing activity (see also similar data on serum in Fig. 3). Since "TOTAL" steroid values included variable and large amounts of "DOC", it seemed dangerous to depend upon the former as a measure of ACTH activity.

Calculation of the ACTH content of the rat pituitary gland from "TOTAL" steroid or "B" release (Fig. 2) gave values approximating 1 i.u. per gland (Table I). Birmingham and co-workers (1956) have recently published similar data, although these investigators claimed that glacial acetic acid was more effective than dilute (0·01N) HCl in extracting ACTH from the rat adenohypophysis. Using 0·1N-HCl as originally recommended by Sydnor and Sayers (1954), we did not find this to be the case as long as equivalent volumes of extraction fluid and thorough homogenization were employed. The above values (c. 1 i.u. per gland) were 10 to 20 times those reported by Burns and co-workers (1949), Gemzell and colla-

similar to those reported by the latter investigators were obtained when rat pituitary extracts were assayed by measuring adrenal ascorbic acid decline *and* corticosteroid release *in vivo* in hypophysectomized rats. In contrast, purified ACTH-protein (Li, Evans and Simpson, 1943) obtained from sheep pituitary was equally effective by both the *in vivo* and *in vitro* assays (Table I). Sheep α-corticotrophin (Li *et al.*, 1955)

release of steroids by the rat adrenal gland *in vitro* (Fig. 2). Pituitary glands were obtained from adult male Sprague-Dawley rats under light Nembutal (pentobarbital sodium)

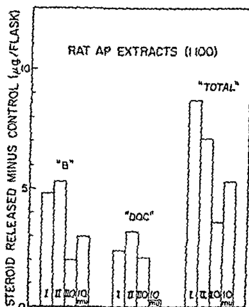


FIG. 2 The influe... hyp

Ti... the... fresh... and one whic... had been allowed to remain for 2 days

anaesthesia. The animals weighed ...

... the same neutralized extract as II, allowed to remain at room temperature for 2 days. This and succeeding charts depict (in contrast to Fig. 1) only the

is the considerable lack of specificity of the *in vitro* procedure (*vide infra*). This non-specificity appears to be especially great where the ACTH present constitutes but a small fraction of the total protein in the sample. The explanation for the somewhat reduced activity of sheep α-corticotrophin *in vitro* was not immediately apparent, particularly in view of the fact that oxycellulose processing of rat and hog ACTH did not seem to alter the original *in vitro* activity of these materials (*vide infra*) However, the latter conclusion was not firmly established from the results obtained. It is possible that ACTH peptide must be linked to protein (perhaps homologous and specific protein) for its full action (*vide infra*; cf. also, Ingle and Li, 1952).

Corticosteroid-releasing Activity in Blood

Assays of rat blood preparations revealed that corticosteroid release by rat adrenal tissue *in vitro* was markedly stimulated by protein or protein-bound substances other than ACTH. All blood samples were obtained from male Sprague-Dawley rats (from our own breeding colony), weighing 200 to 300 g. Light Nembutal (pentobarbital sodium) anaesthesia was employed for the exsanguination procedure. Sera obtained from normal, hypophysectomized, and adrenalectomized rats were all equally effective in evoking the release of "B", "DOC" and "TOTAL" steroids (Fig. 3). Plasma produced equivalent results (Table I). Compared with U.S.P. standards, ACTH-like activity in rat plasma or serum varied between 2·4 to 2·9 i.u./100 ml. (Table I), no difference being noted among the three groups of animals studied. Storage of normal rat serum at room temperature for 2 days (Fig. 3; NO) was without substantial effect on "DOC" or "TOTAL" steroid release, but eradicated the "B"-secretory response. This procedure presumably resulted in the inactivation of blood ACTH (Pincus, Hechter and Hopkins, 1952) and, also, in the denaturation and splitting of other protein complexes. It will be noted (Fig. 3) that rat serum stimulated a greater release of "DOC" than of "B", that the two steroids together accounted

Table I

BLOOD AND PITUITARY ACTH-LIKE ACTIVITY*†

Material	*Amount*	*Method of assay*		
		"TOTAL" corticosteroid release	*Compound "B" release*	*Ascorbic acid depletion*
		U.S.P. equivalents (i.u.)		
α-Corticotrophin (Li)	1 mg.	9 (3)	7 (3)	15
ACTH-Protein (Li)	1 mg.	1·9 (2)	1·8 (2)	2
Rat anterior pituitary	1 gland	1·2 (4)	1·1 (2)	0·1
Intact rat blood	100 ml.			0 to 0·001
Plasma		2·6 (2)	2·4 (2)	
Serum		2·9 (5)	2·5 (5)	
Oxycel (plasma)		0·23 (2)	0·04 (2)	
Hypox. rat blood (1 week)	100 ml.			0
Serum		2·8 (3)	2·5 (3)	
Oxycel (plasma)		0·29 (3)	0 (3)	
Adrx. rat blood (2 weeks)	100 ml.			0·01
Serum		2·7 (6)	2·5 (6)	
Oxycel (plasma)		0·25 (3)	0·14 (3)	
Adrx. cat serum	100 ml.	0·3 (3)	0·1 (3)	0·01
Bovine plasma	100 ml.	0 (4)	0 (4)	0

* The number of separate assays *in vitro* is shown in parentheses following the average values obtained

† All rat blood samples were collected from animals under light Nembutal anaesthesia

was somewhat less active by the *in vitro* assay than by the *in vivo* procedures, even when dissolved in rat serum immediately prior to testing. Considered together, these observations suggest that the rat adrenal gland *in vitro* may be markedly more sensitive to rat ACTH than to ACTH from other sources. *In vivo*, the rat adrenal gland does not appear to make this differentiation, either with respect to the ascorbic acid response or corticosteroid release. If these deductions are correct, it is apparent that the *in vitro* assay for rat ACTH against heterologous ACTH standards will necessarily yield artificially high values. However, an additional factor contributing to the high values for ACTH of rat origin under these circumstances

and adrenalectomized rats removed about 90 per cent of the capacity of these samples to stimulate "TOTAL" corticosteroid release *in vitro* (Table I). As was the case with the

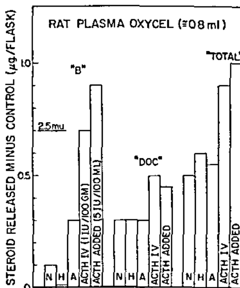


FIG 4 The influence of oxycellulose-purified preparations of various rat plasmas on the release of corticosteroids by rat adrenal tissue *in vitro*.

The effect is shown of adding to the incubation medium 0 2 ml. neutralized oxycellulose extracts equivalent to 0·8 ml. plasma obtained from normal (N), hypophysectomized (H), or adrenalectomized (A) rats. The activity of similarly-treated plasma from normal rats injected with U S P ACTH (1 i u./100 g) and of plasma to which U.S P. ACTH has been added (5 i u /100 ml) is also depicted. For comparison, the effect of 2 5 mu U S P. ACTH on "B" release is recorded See text for further explanation.

original plasma samples, no differences were noted among the three groups with respect either to "TOTAL" steroid release or "DOC" release (Fig. 4). On the other hand, significant differences were observed in the relative abilities of these

for 100 per cent of the extra steroid released (over and above that released in the control flasks), and that, under such circumstances, measurement of "TOTAL" steroid release could be completely misleading as an index of "B" release (cf. results with sample NO). The unreliability even of "B" release as a measure of ACTH was apparent from the fact that

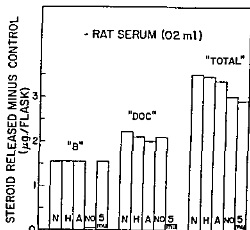


FIG. 3 The influence of various rat sera on the release of corticosteroids by rat adrenal tissue *in vitro.*

removed by the oxycellulose technique of Astwood and coworkers (1951) as adapted by Sydnor and Sayers (1952). The results obtained when this procedure was applied to various rat plasmas are summarized in Fig. 4 and Table I. Oxycellulose treatment of plasma from normal, hypophysectomized,

was obtained from these and other observations that heterologous proteins may actually inhibit corticosteroid release by rat adrenal tissue *in vitro*. If this be true, it further complicates the use of an *in vitro* assay for the measurement of ACTH in heterologous materials.

Localization of ACTH-like Activity in Plasma Protein Fractions

The *in vitro* corticosteroid-releasing activity of rat plasma or serum was found to be quantitatively recovered in the dialysed, lyophilized total proteins, indicating that the active materials were protein or protein-bound. Actually, 1 mg. whole plasma protein produced a significant response. In attempts to localize this activity more specifically and to separate the various types of adrenal-stimulating materials which appeared to be present, the cold ethanol fractionation technique (Roberts and Kelley, 1956) was applied to samples of rat plasma. It was found that most of the *in vitro* corticosteroid-releasing activity of plasma obtained from normal, hypophysectomized, or adrenalectomized rats was concentrated in the β- and γ-globulin fractions This is shown for adrenalectomized rat plasma in Fig 5. Actually, calculation revealed that almost all of the residual "B"-releasing activity remaining after cold ethanol fractionation was present in the β-globulins (Table II), whereas "DOC"-releasing activity was concentrated in the γ-globulins. The albumin and α-globulin fractions were remarkably poor in corticosteroid-releasing activity *in vitro*. Obviously measurements of "TOTAL" corticosteroid release did not differentiate between the "B"- and "DOC"-releasing functions of β- and γ-globulin. Calculations of ACTH-like activity in the plasma proteins of hypophysectomized and adrenalectomized rats based on these results are presented in Table II, where appropriate consideration has been given to the relative amounts of the different protein fractions present in the original plasma as judged by conventional electrophoretic analysis. All of the "TOTAL" corticosteroid-releasing activity could be recovered in the various

preparations to evoke "B" release. Calculation of the ACTH-like activity of plasma from the latter results gave values of 40, 0, and 140 mu. per 100 ml. for normal, hypophysectomized, and adrenalectomized rats, respectively (Table I). The first and last values were still 10 to 20 times (or more) greater than those obtained employing the adrenal ascorbic acid assay (Gemzell *et al.*, 1951; Sydnor and Sayers, 1952, 1954; Barrett and Hodges, 1956). However, it will be recalled that this was the same order of difference in the apparent sensitivity of the *rat adrenal gland in vitro to pituitary ACTH of rat origin* as contrasted with ACTH of other origin (*vide supra*). It would appear, then, that the oxycellulose technique may remove the majority of the non-specific materials in rat plasma which stimulate "B" release *in vitro*.

Homologous blood preparations were apparently not extensively tested *in vitro* by the McGill group. However, experiments were cited in which citrated whole blood was employed as the incubation medium with the resultant inhibition of corticosteroid release (Saffran and Bayliss, 1953). Haynes, Savard and Dorfman (1954) reported that citrated beef blood, beef plasma, or phosphate buffer served equally well as media for studying the action of ACTH on corticosteroidogenesis by beef adrenal slices, and that bovine plasma fraction III had no effect on this process. In contrast, Hofmann and Davison (1954) observed that heparinized rat blood stimulated the output of corticosteroids by rat adrenal tissue *in vitro*. It may be that large amounts of citrate are toxic to adrenal tissue or, by complete removal of calcium, may depress steroid release even in the absence of ACTH (cf. however, Birmingham *et al.*, 1953). In this connection, it should be noted that the present studies were conducted with serum or plasma added to a calcium-fortified incubation medium (cf. McKerns and Nord- [illegible]

adrenalectomized cat serum, bovine plasma). The impression

enough, albumin prepared from the plasma of adrenalectomized rats, although substantially devoid of corticosteroid-releasing activity *in vitro* (Fig. 5), was extremely active by the *in vivo* assay procedures (Table II). In fact, it appeared that

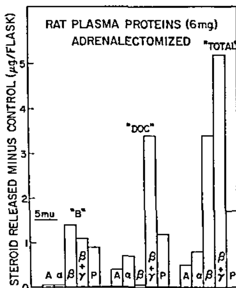


FIG. 5. The influence of plasma proteins from adrenalectomized rats on the release of cortico-

A, albumin; α, α-globulin, β, β-globulin, β+γ, a mixture of 65 per cent β-globulin and 35 per cent γ-globulin. For comparison, the effect of 5 mu U.S.P. ACTH on "B" release is also depicted.

potentiation may have occurred, but this was not established. It has previously been reported that corticotrophin A bound to bovine albumin is more effective than the free hormone in the adrenal ascorbic acid assay (Brown *et al.*, 1955).

The specificity of the *in vivo* ACTH assay methods employed appeared to be established by the fact that none of the

Table II

ACTH-LIKE ACTIVITY IN RAT PLASMA PROTEIN FRACTIONS*

Material	*Method of assay*		
	"TOTAL" corticosteroid release in vitro†	*Compound "B" release in vitro*†	*Ascorbic acid depletion and corticosterone release in vivo*†
	U.S.P. equivalents (I.U.)		+ or −
Hypox. rat plasma (1 week)			
Oxycel extract	0 29	0	
Total protein	2·20	2 05	−
Albumin	0	0	−
α-Globulin	0·22	0	−
β-Globulin	1 03	0 83	−
γ-Globulin	0 88	0	−
Adrx. rat plasma (2 weeks)			
Oxycel extract	0 25	0 14	
Total protein	3·06	2 80	−
Albumin	0·45	0	+
α-Globulin	0·46	0	±
β-Globulin	0 97	0 70	±
γ-Globulin	1 07	0 07	−
Normal rat plasma U.S.P. ACTH added (5 I.U./100 ml.)			
Oxycel extract	4·45	4·02	
Total protein	9 82	9 38	+
Albumin	5 54	4 49	+
α-Globulin	0 89	0·13	+
β-Globulin	0 74	0·65	+
γ-Globulin	0·90	0·04	+

* [illegible]

plasma fractions. Only about 25 per cent of the "B"-releasing action present in the dried plasma could be recovered, and this almost exclusively in β-globulin. However, it should be noted that assays of 10 ml. adrenalectomized rat plasma equivalents of the β-globulin fraction by the ascorbic acid decline and *in vivo* corticosteroid-release techniques failed to reveal more than a trace of ACTH activity in this fraction: Remarkably

Sayers (1952), Sydnor (1955), and Parrott (1955) employing the oxycel procedure and adrenal ascorbic acid assays. ACTH added *in vitro* appeared in all of the plasma fractions on the basis of the *in vivo* assay results (Table II). However, the *in vitro* assay gave conflicting results. Thus, the added ACTH

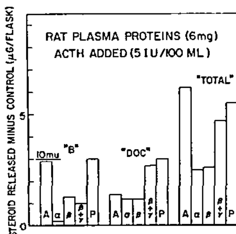


FIG. 6 The influence of proteins from normal rat plasma to which U.S.P. ACTH had been added on the release of corticosteroids by rat adrenal tissue *in vitro*.

seemed to be localized almost exclusively in the albumin fraction judging from the extra "B"-releasing activity of the various plasma fractions (Fig. 6) over and above that found in similar fractions prepared from normal, hypophysectomized, or adrenalectomized rat plasma (cf. Fig. 5). Comparable data were obtained on plasma from animals given ACTH intravenously 1 minute before exsanguination (Fig. 7).

From the *in vivo* assay results, it may be tentatively concluded that endogenous circulating ACTH in the rat is

plasma protein fractions obtained from the hypophysectomized rat possessed activity in these procedures. In contrast, significant corticosteroid-releasing activity *in vitro* was found in the β- and γ-globulin fractions of plasma from hypophysectomized rats (Table II). It may be concluded that the *in vitro* procedure is not only non-specific, but, in addition, may be insensitive to ACTH which is associated with relatively large amounts of other proteins. It may be noted here that human and bovine plasma protein fractions were completely inactive *in vitro*. No attempt was made to determine whether this inactivity was due to lack of sensitivity of the rat adrenal to non-rat ACTH, to a "masking" effect, or to the presence of heterologous protein, etc.

Experiments were also carried out on the recovery of U.S.P. corticotrophin added to normal rat blood *in vitro* or injected intravenously into normal rats. Attempts were made to determine the localization of the exogenous ACTH in the plasma protein fractions under these circumstances. In the *in vitro* addition experiments, the equivalent of 5 i.u. ACTH was added to 100 ml. freshly-prepared rat plasma and appropriate aliquots of the latter were immediately subjected either to the oxycellulose purification technique or to cold ethanol fractionation. In the *in vivo* experiments, the equivalent of 1 i.u. ACTH per 100 g. body weight was injected into normal rats via the saphenous vein and the animals were exsanguinated 1 minute later. The plasma was prepared immediately and subjected to the oxycellulose and cold ethanol fractionation techniques. The *in vitro* corticosteroid-releasing activity of both oxycel preparations is shown in Fig. 4 and that of the ethanol-fractionated plasma proteins in Figs. 6 and 7. The results obtained after the *in vitro* addition of ACTH to plasma are summarized in terms of ACTH equivalents in Table II. It will be noted (Table II) that the oxycel preparation of plasma to which ACTH had been added *in vitro* appeared to contain about 80 per cent of the added hormone, as judged from "B"-releasing activity *in vitro*. Comparable recoveries have been reported by Sydnor and

mones (e.g., thyroxine, oestrogen, thyrotrophin) may be transported in association with one of the plasma proteins, but when secreted in excessive quantities or introduced exogenously tend to "spill over" into association with other plasma proteins (cf. Robbins, Petermann and Rall, 1955; Roberts and Szego, 1955; Roberts and Leyey, unpublished).

Summary and Conclusions

Detailed studies have been carried out on the release of corticosteroids by rat adrenal tissue *in vitro* (Saffran procedure) in response to various ACTH preparations and to rat adenohypophysial extracts and blood fractions. The two steroids released in largest quantities under almost all circumstances had the chromatographic, spectrophotometric, and staining properties of corticosterone ("B") and 11-desoxycorticosterone ("DOC"). The release of "B" and of "TOTAL" corticosteroids (absorbing maximally at 240 mμ.), but not of "DOC", was proportional to the amount of purified ACTH added to the incubation medium.

Dilute acid extracts of rat adenohypophyses, assayed by the Saffran technique, yielded ACTH values about fifteen times greater than those obtained with the Sayers assay, employing in both procedures ACTH reference standards of porcine or sheep origin. Sheep ACTH-protein (Li) assayed identically by both procedures; α-corticotrophin (Li) was somewhat less effective by the Saffran technique. These results suggested that the release of corticosteroids by the rat adrenal gland *in vitro* was markedly responsive to rat ACTH and much less responsive to ACTH from other sources.

Rat plasma and serum samples were extremely active in evoking the release of corticosteroids *in vitro*. Approximately equal amounts of "B" and "DOC" were secreted under these circumstances. No differences were observed among sera or plasma obtained from normal, hypophysectomized, and adrenalectomized rats. Application of the oxycellulose technique removed much of the "B"-releasing activity from the blood of normal and adrenalectomized animals and all of

transported mainly or exclusively in the albumin fraction of the plasma proteins. Exogenous hormone also appears to bind, in part, to the albumin but, when present in large quantities, may bind to certain of the other plasma proteins.

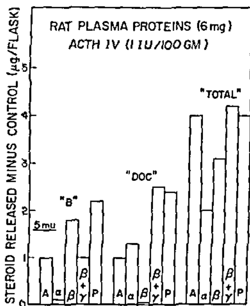


FIG. 7. The influence of plasma proteins from rats injected intravenously with U.S.P. ACTH on the release of corticosteroids by rat adrenal tissue *in vitro*.

The equivalent of 1 I.U. U.S.P. ACTH per 100 g body weight was injected intravenously 1 minute before exsanguination. The plasma was rapidly prepared and subjected to the cold ethanol fractionation procedure. See text and legend to Fig 5 for further explanations

These interpretations must be made with caution, however, since *endogenous* protein-bound ACTH was measured only in the adrenalectomized rat, where elevated circulating levels were present. In addition, the *exogenous* ACTH was not of rat origin. Evidence has been accumulating in the literature that under normal circumstances various endogenous hor-

BIRMINGHAM, M. K., ELLIOTT, F. H., and VALERE, P. H.-L. (1953). *Endocrinology*, 53, 687.
BIRMINGHAM, M. K., KURLENTS, E., ROCHEFORT, G. J., SAFFRAN, M., and SCHALLY, A. V. (1956). *Endocrinology*, 59, 677.
BROWN, R. A., MOYER, A. W., DAVIES, M. C., and COX, H. R. (1955). *Arch Biochem Biophys.*, 58, 68.
BURNS, T. W., MERKIN, M., SAYERS, M. A., and SAYERS, G. (1949). *Endocrinology*, 44, 439.
BUSH, I. E. (1952). *Biochem. J.*, 50, 370.
BUSH, I. E. (1953) *J. Endocrin.*, 9, 95.
EISENSTEIN, A. B. (1956). *Proc Soc. exp. Biol*, *N.Y.*, 91, 657.
[illegible] V., and SCHONBAUM,
[illegible] *J. Biochem Physiol.*,
[illegible]
GEMZELL, C. A., VAN DYKE, D. C., TOBIAS, C. A., and EVANS, H. M. (1951). *Endocrinology*, 49, 325.
HAYNES, R., SAVARD, K., and DORFMAN, R. I. (1954). *J. biol Chem*, 207, 925.
HOFMANN, F. G., and DAVISON, C. (1954) *Endocrinology*, 54, 580.
INGLE, D. J., and LI, C. H. (1952). *Proc. Soc. exp. Biol.*, *N.Y.*, 79, 128.
LI, C. H., EVANS, H. M., and SIMPSON, M. E. (1943). *J. biol. Chem.*, 149, 413.
LI, C. H., GESCHWIND, I. I., DIXON, J. S., LEVY, A. L., and HARRIS, J. I. (1955). *J biol. Chem*, 213, 171.
LIDDLE, G. W., ISLAND, D., RINFRET, A. P., and FORSHAM, P. H. (1954). *J. clin Endocrin.*, 14, 839.
LONG, C. N. H. (1946). *Recent Progr. Hormone Res.*, 1, 99.
MCKERNS, K. W., and NORDSTRAND, E. (1955) *Canad. J Biochem. Physiol.*, 33, 209.
NELSON, D. H., and HUME, D. M. (1955) *Endocrinology*, 57, 184.
PARROTT, D. M. V. (1955) *J. Endocrin*, 12, 120.
PINCUS, G., HECHTER, O., and HOPKINS, T. (1952) *J. clin. Endocrin.*, 12, 920
[illegible]
[illegible] *Endocrinology*, 52, 140
[illegible] *Endocrinology*, 56, 523.
[illegible] *Canad. J. Biochem. Physiol*,
[illegible]
SAFFRAN, M., SCHALLY, A. V., and BENFEY, B. G. (1955) *Endocrinology*, 57, 439.
SAYERS, G., and SAYERS, M. A. (1948). *Recent Progr. Hormone Res*, 2, 81.
SAYERS, M. A., SAYERS, G., and WOODBURY, L. A. (1948). *Endocrinology*, 42, 379
SINGER, B., and STACK-DUNNE, M. P. (1955). *J. Endocrin*, 12, 130

this activity from the blood of hypophysectomized rats. In the former instances, the ACTH-like activity remaining was still about fifteen times that reported using the ascorbic acid assay, apparently due, in part, to the differential sensitivity of the rat adrenal *in vitro* to rat ACTH and to ACTH from other sources (assay standards).

The "B"-releasing activity of rat plasma was concentrated in the β-globulins, whereas "DOC"-releasing activity was located in γ-globulin. No differences were observed in these activities among fractions obtained from normal, adrenalectomized, or hypophysectomized animals. In contrast, U.S.P. ACTH added to rat serum *in vitro* or injected intravenously appeared almost exclusively in the albumin fraction, as judged from "B"-releasing activity *in vitro*. Limited assays of these various plasma protein fractions in the hypophysectomized rat (adrenal ascorbic acid decline and corticosteroid release into the adrenal venous effluent) revealed no ACTH activity in plasma fractions of hypophysectomized rats, endogenous activity in the albumin fraction of plasma from adrenalectomized rats, and exogenous activity in all of the plasma fractions. It is concluded that the *in vitro* corticosteroid-release assay for ACTH may be both non-specific and non-responsive in the presence of interfering protein or protein-bound substances.

Acknowledgement

The author wishes to acknowledge the valuable assistance in these studies of Mrs. E. Cheever, Mrs. K. Marso, Mrs. L. Monroe, and Dr. M. A. Slusher. Samples of pure corticosteroids were kindly provided by Dr. E. Alpert of Merck, Sharp and Dohme, Rahway, N.J. Samples of ACTH-protein and α-corticotrophin were generously made available by Dr. C. H. Li, University of California, Berkeley.

REFERENCES

ASTWOOD, E. B., RABEN, M. S., PAYNE, R. W., and GRADY, A. B. (1951). *J. Amer. chem. Soc.*, 73, 2969.
BARRETT, A. M., and HODGES, J. R. (1950). *J. Endocrin.*, 13, 391.
BEIGELMAN, P. M., SLUSHER, M. A., SLATER, G. G., and ROBERTS, S. (1956). *Proc. Soc. exp. Biol., N.Y.*, 93, 608.

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However, at this point I am rather inclined to think that these results

specific surface-stimulating materials in rat plasma proteins which result in steroid release

Vogt. What is the ratio of cortisone to corticosterone which you find?

Roberts After ACTH addition the ratio is probably about 1 . 10.

Roberts· Presumably the adrenal gland *in vivo* is normally exposed to these "stimulatory" substances and therefore normal steroid secretion is dependent upon their presence Calculation would probably reveal that the amount of corticosteroid secreted *in vitro* under the most favourable circumstances does not exceed the best estimates of the normal rate of secretion *in vivo*.

Heller· Did I understand you to say that the levels of true ACTH were higher in the rat than in other species?

Roberts· No. I don't believe that is the case However, it does appear that the rat adrenal may be more sensitive to rat ACTH than to ACTH of other origin.

Randle· How do the activities per mg. of non-specific protein and

corticosteroids from the adrenals of hypophysectomized *dogs.* Does this procedure have the same limitations as those you have described for Saffran's method?

Roberts The technique of Hume and Nelson, as you know, depends on the release of 17-hydroxycorticosteroids into the adrenal venous

SLUSHER, M. A., and ROBERTS, S. (1956). *Proc. Amer. Physiol. Soc., 8th Ann. Mtg*, p. 138.
STACK-DUNNE, M. P., and YOUNG, F. G. (1954). *Annu. Rev. Biochem.*, 23, 405.
SYDNOR, K. L. (1955). *Endocrinology*, 56, 204.
SYDNOR, K. L., and SAYERS, G. (1952). *Proc. Soc. exp. Biol., N.Y.*, 79, 432
SYDNOR, K. L., and SAYERS, G. (1954). *Endocrinology*, 55, 621.
TOUCHSTON, J. C., and HSU, C.-T. (1955). *Analyt. Chem.*, 27, 1517.

DISCUSSION

Farrell I should like to hear Dr. Roberts comment on the bearing of his findings to the non-specificity of the method What do you think your findings mean with regard to the results of Saffran and his co-workers on ACTH-releasing factors in Pitressin or in hypothalamic material?

Roberts. I believe that any results obtained by the *in vitro* ACTH assay technique must be re-evaluated in the light of the present findings. As I have tried to point out protein or protein-bound substances other than ACTH are capable of stimulating the release of corticosteroids *in vitro*. We have also reason to believe that the release of ACTH by pituitary tissue *in vitro* can be induced non-specifically. It would appear therefore, that the significance of the work on pituitary-stimulating factors using these procedures is in considerable doubt.

Samuels Dr. Roberts, did you try any detergents in this system, since it would be easy to have the type of result indicated here if you had some detergent action on the surface of these quartered cells which would simply release material from them?

Roberts: No, we did not investigate the effects of detergents in this system I agree with you that many other substances might have this effect. However, it should be recalled that as little as 1 mg rat serum protein in the presence of 2 rat adrenals *in vitro* will cause a very substantial release of steroid

Vogt: In connection with Dr. Samuels' question, if I understand rightly, these methods are not just estimating a release of stored corticoids, because a release would not account for the large quantities found. There the detergent would not act by releasing what is there but would be bringing into contact substrate and enzymes.

Roberts: You are quite right, Dr. Vogt. There is a pre-incubation period which has been demonstrated to remove the major portion of the steroids present in the adrenal tissue initially.

Vogt: One question which worries me a great deal is the release of cortisone under those conditions because the living rat does not ever seem to produce cortisone, not even when the quantities of corticosterone are very large indeed. Now do you think that there is also some breakdown of a barrier between an enzyme and a substrate involved? What are the quantities you actually find?

MORPHOLOGICAL CHANGES IN THE ADRENAL CORTEX IN RELATION TO CONCENTRATION OF STEROIDS IN ADRENAL VEIN BLOOD

MARTHE VOGT

Pharmacological Laboratory, University of Edinburgh

THIS paper deals with information on the functional state of the adrenal cortex which can be derived from the chemical estimation of corticoids in adrenal vein blood. I shall confine myself to experiments on the anaesthetized rat subjected to the severe operative stress of a laparotomy and cannulation of the left renal vein. Under these conditions, enough ACTH is released from the anterior pituitary to cause the maximal rate of secretion obtainable from the adrenal cortices. This is shown by the observation that infusion of exogenous ACTH in such experiments does not increase secretion. What is being measured, then, is not resting secretion or mildly accelerated secretion, but the maximal secretion rate of which the gland is capable.

The technique (Vogt, 1955) consists of introducing cannulae into the trachea, the left femoral vein and, if blood pressure records are required, into the left femoral artery of rats weighing approximately 250 g. After an intravenous injection of heparin (1,000 i u /kg.), the collecting cannula is introduced into the left renal vein which has been tied at the renal hilum and at its entry into the vena cava. The blood is collected into cooled, siliconed tubes and centrifuged; the plasma is extracted with organic solvents, and the purified extracts are chromatographed in one of Bush's solvent mixtures (Bush and Sandberg, 1953). Since, in the rat, corticosterone is the main adrenal corticoid, the estimation of secretion rate was carried out by localizing the corticosterone region by u.v. absorption, eluting the compound and estimating it colorimetrically,

affluent of the hypophysectomized dog. The method is sensitive approximately 1 mu. ACTH. I heard from Dr. Ganong recently th substances other than ACTH may cause the release of 17-hydroxyc ticosteroids under these circumstances.

Tait: Do these crude extracts of blood have any effect on rat adre homogenates? Presumably purified ACTH would not have an effect such preparations.

Roberts: I don't know; we have not studied rat homogenates. Ho ever, Rosenkranz has recently reported that ACTH will cause cor costeroid release in adrenal homogenates.

Tait. I think it is rather an isolated observation.

As regards this question of disintegration which may also be releva to Dr Astwood's query, I feel that these preparations may be lial to be broken up by added material. If I remember rightly, they a incubated for an hour before the ACTH is added and they are rath thick slices Bearing in mind the concept of Hechter that a hom genate is equivalent to an ACTH-stimulated tissue, disintegration m result in an ACTH effect.

Roberts: It all depends upon what you call "thick". The rat adre weighs about 12 mg Since it is divided into four equal portions in the studies, each portion weighs about 3 mg. That is not very much!

Tait What would be the maximum thickness?

Roberts: I would have to measure it, but it is rather small.

Tait: As regards the cortisone, I think we probably had a simil compound produced in ox adrenal tissue preparations, which on e tensive fractionation we found was not cortisone. This compou actually was increased on incubation, but cortisone itself of which the was a little, was not increased on incubation.

borators (see Hertz *et al.*, 1955) to produce a variety of effects on the endocrine system: it has progestational activity, it is a goitrogen and it causes a cholesterol deposition in the adrenal cortex unequalled by any other substance, including ACTH, and not accompanied by changes in the cholesterol concentration of blood or liver. Injected intravenously into dogs (Hertz *et al.*, 1955) or rats (Vogt, 1956), it depresses cortical secretion, but the general toxicity of intravenous

Table I

EFFECT OF PROLONGED AND OF BRIEF, INTENSIVE TREATMENT WITH ACTH ON SIZE AND CORTICOSTERONE SECRETION OF THE RAT ADRENAL

No. of rats	*Treatment*	*Adrenal weight mg./kg. body wt.*	*Corticosterone secreted (mean ± S E. of the mean)*	
			µg / g gland/ min	*µg./adrenal/hr./ kg. body wt.*
11	None	62 6 ± 2 4	25 4 ± 1 5	95 6 ± 9 5
7	1 i u daily* for 10–14 days	90 0 ± 3 3	25 9 ± 3 9	137 1 ± 17 5
8	16–44 i u † within 36 hr.	97 5 ± 4 9	17 0 ± 1 7	99 7 ± 11 1

Adrenal blood collection in urethane anaesthesia (1 5 kg /g. subcutaneously).

* Long action ACTH ("Cortrophin Z", Organon) subcutaneously
† Long-action ACTH 12-hourly, soluble ACTH 4-hourly during daytime, all injections subcutaneously

doses is very high, and the results therefore are not necessarily comparable to the effects of an oral administration of the drug over several days. Only the latter causes the hypertrophy and accumulation of lipids, the functional equivalent of which this investigation was trying to determine.

Fig. 1 shows the results of tests of the functional capacity of rat adrenals after 2, 4 and 14 days of amphenone by mouth. Secretion per g tissue is normal or even slightly above average; since hypertrophy of the glands is large and rapid, secretion per single gland is much above normal, even when only two doses of amphenone have been administered.

using a modification of the reaction with blue tetrazolium described by Mader and Buck (1952). The colour was measured in a Unicam spectrophotometer at 520 mμ. The results are expressed in μg. secreted per g. gland per min., or in μg. secreted per gland per kg. bodyweight per hour.

Morphological changes in the rat adrenal are elicited by many drugs and hormones, hypertrophy of the tissue and loss or accumulation of sudanophilic lipids being the effects most commonly produced. Obviously, many of these changes are simply due to the release of ACTH, brought about by direct or, usually, indirect effects of the drug on the structures which control pituitary secretion. Some substances, however, whilst depending for their action on the presence of the anterior lobe, have additional direct effects on adrenal metabolism, and such substances are of special interest in this work.

Since so many drugs act on the adrenal cortex through the release of ACTH, the effect of injections of ACTH for variable periods previous to the collection of blood will be shown first (Holzbauer and Vogt, to be published). The results obtained are quite different, depending on whether the hormone is injected over a prolonged period of time or precipitously. The slow treatment causes lipid accumulation in all cortical layers, the rapid treatment moderate lipid depletion of the zona fasciculata.

Table I shows that adrenal enlargement is approximately the same in both instances; but, whereas secretion of corticosterone per g. adrenal is normal in the lipid-rich gland, it is diminished in the lipid-depleted gland subjected to a brief period of heavy dosage. Conversely, secretion per whole gland is increased after slow, but remains normal after rapid administration of ACTH. The inference is that in both experiments the glands have produced new tissue, but a longer period of time is necessary for the production of fully functional tissue than for a mere increase in tissue mass.

The second example chosen for illustration is that of "Amphenone B" [1 : 2-bis(*p*-aminophenyl)-2-methylpropane-1-one]. This substance was shown by Hertz and his colla-

· Observations in patients suffering from adrenal carcinoma and treated orally with amphenone for a few days (Thorn *et al*, 1956), were in sharp contrast to the findings in the rat: a fall in circulating and in excreted 17-hydroxycorticoids was observed during the administration of the drug, which, incidentally, was poorly tolerated. Perhaps there is some connection between these two facts.

It has been known for many years that stilboestrol and

Table II

EFFECT OF HEXOESTROL (0 4 MG /KG. SUBCUTANEOUSLY PER DAY IN OIL) ON CORTICOSTERONE SECRETION OF RAT ADRENALS

No of rats	*Duration of treatment (days)*	*Corticosterone secreted (mean ± S E. of the mean)*	
		μg /g. gland/min	μg /adrenal/hr /kg body wt.
11	None	25 4 ± 1 5	95 6 ± 9 5
8	3–15	6 9 ± 0 5	32 3 ± 3 3
5	2	9 7 ± 2 1	36 0 ± 8 2

Adrenal blood collection in urethane anaesthesia (1 5 g./kg subcutaneously)

hexoestrol cause a loss in adrenal lipids (Vogt, 1945). In contrast to the fleeting effect of injections of ACTH, this loss increases with prolongation of treatment and is only reversible when the administration of the oestrogen is stopped. The functional state of such lipid-free adrenals has been a matter for much speculation, but the method described in this paper provides a means of settling the question experimentally (Vogt, 1955)

Table II illustrates some of the results. Secretion per g. gland is very seriously depressed, and a period of only two days is sufficient to produce the effect. Adrenal lipids are also appreciably reduced at that time. In order to check the suggestion that the fall in secretion followed an initial increase, estimations were also made after a single injection, the time

Contrary, then, to what sometimes has been suggested, the lipid accumulation caused by amphenone does not indicate arrest of corticoid synthesis at the cholesterol stage. The drug produces an enlarged gland made up of fully functional

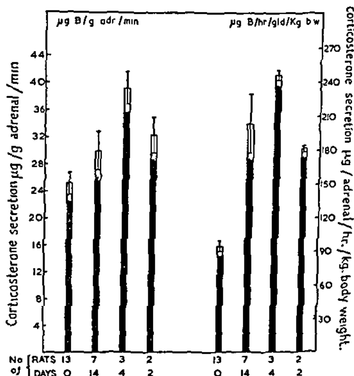


FIG. 1. Effect of amphenone (0 2 g./kg. daily by mouth) on corticosterone secretion of rat adrenal.
Abscissae (bottom figures): duration of treatment in days.

tissue. Attempts at producing an equally large lipid-filled gland in the brief period of between two and four days by means of ACTH injections failed. This suggests that the changes produced by amphenone in the rat adrenal cannot be explained solely on the basis of an increased secretion of ACTH; some direct effect on adrenocortical metabolism may be a contributory factor.

state of the adrenal cortex following treatment with hexoestrol would thus be analogous to the state of the thyroid after the administration of thiouracil.

Acknowledgement

"Amphenone B";
supply of ACTH

REFERENCES

BOYD, G. S., and MCGUIRE, W. B. (1956). *Biochem. J.*, **62**, 19P.
BUSH, I. E., and SANDBERG, A. A. (1953). *J. biol. Chem.*, **205**, 783.
GEMZELL, C. A. (1952). *Acta endocr., Copenhagen*, **11**, 221.
HERTZ, R., TULLNER, W. W., SCHRICKER, J. A., DHYSE, F. G., and HALLMAN, L. F. (1955). *Recent Progr. Hormone Res.*, **9**, 119.
HOLZBAUER, M. (1956). *J. Endocrin.*, **14**, XXVI.

DISCUSSION

interval between injection and experiment varying between 3·5 and 24 hours. Secretion was invariably found to be at or below the normal level. Controls showed that no other cortical compound was replacing the corticosterone in the hexoestrol-depressed adrenal. Stores of corticosterone, estimated in adrenals of normal rats and of rats given hexoestrol, the two groups of animals having been killed under precisely identical conditions, were also found to differ (Holzbauer, 1956): per g. tissue, the corticosterone content of the adrenals of normal rats was about twice that of hexoestrol-treated rats.

The mechanism of the decrease in corticosterone secretion is considered to be an inhibition of cholesterol synthesis. The following points support such a view:

(1) After hexoestrol, adrenal and blood cholesterol concentrations are low.

(2) Liver slices of rats treated with hexoestrol synthesize cholesterol from ^{14}C-labelled acetate at a greatly reduced rate (Boyd and McGuire, 1956).

(3) Plasma from normal rats, but not plasma from hexoestrol-treated rats, immediately restores to normal the corticosterone secretion from adrenals which have been inhibited by hexoestrol. There is about ten times as much cholesterol in normal rat plasma as there is in plasma of rats to which hexoestrol has been given.

(4) When rats, previously injected with hexoestrol, are fed amphenone whilst the hexoestrol injections are being continued, the corticosterone secretion of their adrenals reverts to normal figures per g. gland. The lipids, previously absent from the adrenals, reappear in the cortical tissue; since the major compound of these lipids is cholesterol (and its esters), restoration of function appears to be due to the renewed availability of cholesterol as substrate of corticosterone synthesis.

The adrenal hypertrophy and hyperaemia found after hexoestrol are probably consequences of the increase in circulating ACTH demonstrated by Gemzell (1952) and presumably caused by lack of circulating cortical steroids. The

these experiments (which were done in hypophysectomized rats) was

stration that under those conditions growth hormone is not, in fact, ultrafiltrable It is a substance which has a relatively low molecular

relatively minor scale.

increase in stainable lipid obtained when growth hormone is given in addition to large amounts of corticotrophin, which by itself severely

where you got changes with growth hormone, or growth hormone plus corticotrophin?

large doses of corticotrophin and growth hormone.

metabolism cannot be stimulated by ACTH after hexoestrol; injection of ACTH to hexoestrol-treated rats does not improve their performance. Further, ACTH content of the blood is permanently raised in hexoestrol-treated rats, and this seems to point to a deficiency in the adrenals themselves.

Morris: I wondered if there was something that behaved rather similarly to amphenone but which was a naturally occurring substance, of any nature—it is impossible to say of what nature. The effects were so gross. A preparation that had approximately 1 u. per mg. adrenal ascorbic acid depleting activity would double the weight of adrenals within three days

Vogt: Do you know whether these were yellow adrenals or red adrenals?

Vogt: The effect of amphenone has been shown by Hertz and Tyler to be absent in ACTH-maintained hypophysectomized rats. The effect of hexoestrol was tested many years ago (when there was no ACTH available) only on hypophysectomized, not ACTH-maintained, rats, and no depletion of sudanophilic lipids was seen. The combined effect of amphenone and hexoestrol has not been tested on hypophysectomized rats at all.

The animals which had hexoestrol plus amphenone were not in very good shape. They would not die spontaneously, but if you then had to do this operation it was not too easy to keep them alive, one of the

Migeon: I had in mind our work on dying human patients (1955. *J. clin Endocrin Metab.*, 15, 1001) where we found that the increase in free plasma 17-hydroxycorticosteroids was due to a slow metabolism of cortisol rather than an increased production of hormone

Vogt: This point here does not arise if you take adrenal vein blood.

Migeon: The steroid content of adrenal vein blood is made up of the steroids which were present in the adrenal artery, that is to say peripheral blood, plus the steroids secreted by the gland. This is why it would be important to compare levels in peripheral and adrenal vein blood.

Vogt: Yes, but I think that quantity would never have occurred in these animals in peripheral blood. I tested arterial blood after 14 days of amphenone and found no corticosterone in a volume of plasma twice that used for estimation in adrenal effluent.

Astwood: Dr. Vogt, have you tested corticotrophin administered in some really good delaying vehicle such as the Bruce and Parkes oil and

Vogt. We gave the long-acting material every 12 hours, do you think that is not sufficient?

Astwood: I would not know

Vogt: I was struck by this statement by Hertz on the amphenone

inhibition of adrenal secretion or a loss of adrenal cholesterol with

Vogt: When you studied the morphological changes how long were your injection times?

Slack-Dunne: Most of that work was injection for three days. The rats were about 3 5 weeks after hypophysectomy and were then injected for three days. There were some rats that Bush and I had worked on that had been treated with growth hormone for the week directly after hypophysectomy

Sa [illegible] retion

Vog [illegible] ut the

with [illegible] know

mate [illegible] whether the

whether this is really a specific effect on the adrenals or whether the [illegible]

[illegible] exoestrol [illegible] l-treated [illegible] l plasma [illegible] n't know [illegible] you the [illegible] troduced

[illegible] and then

[illegible] ss in that

very short time. [illegible] hormone are

[illegible] man

[illegible] oxy- [illegible] e. I [illegible] one [illegible] nen- [illegible] ter- [illegible] the

[illegible] r its [illegible] cent [illegible] that [illegible] none [illegible] gues [illegible] ving [illegible] tical

[illegible] the

[illegible] peripheral circulation of amphenone-treated rats?

Vogt: Yes.

Migeon: Could these rats have been so sick that they were not far from dying?

Vogt: No, I would not say so, not the rats given amphenone daily.

GENERAL DISCUSSION

von Euler: Dr. Farrell, have you any data on the effects of oxytocin on the release of ACTH and also of substance P ?

Farrell: Yes, both substances are of considerable interest. Oxytocin has been tried, and it is inactive. Substance P has not been tested but I believe Dr. Sayers plans to test it in the near future.

Pearlman I am curious about a point which Dr. Pitt-Rivers

of oestrogen is rather low in the initial states of gestation, but progressively increases. Now what is the dose response in the non-pregnant patients, with respect to PBI levels and the dose of oestrogen administered? It would appear from Dr. Pitt-Rivers' talk that maximal stimulation of PBI was obtained with the relatively small amounts of oestrogen which are produced in early pregnancy.

Pitt-Rivers: I don't exactly remember Engstrom's dose-response

dosage corresponds roughly to the oestrogen output in late pregnancy rather than to that in early pregnancy, and I am curious to know what sort of response you might get with, let us say, one-tenth the dose of hexoestrol.

Pitt-Rivers I think that Ingbar indicated that you could give lower doses of diethylstilboestrol to affect the protein-binding of thyroxine; there may be no parallelism, but it happens that PBI and protein binding are raised in pregnancy. If you artificially produce a high oestro

in protein binding

PBIs in preganc**y**

further consideration.

Pearlman Did you say there is an increased calorific effect

ris

oestrogens. With the dose that is used in rats you certainly cannot produce it, and in the rabbit I have tried up to twenty-five times the dose used in the rat and nothing happens at all. So far, I am quite prepared to believe that the rat, of all the laboratory animals, is the only one that responds with inhibition. The second point is that even in the rat, I

... getting into the

... we can get

... to collect

adrenal vein blood from a conscious rat without producing any stress.

...

cutting its head off—you find, irrespective of what the pretreatment was, very little corticosterone in the glands. If you take another rat

...

indication that there is, under conditions of ordinary life, an increased secretion in spite of a decreased capacity for maximal secretion.

Stack-Dunne· Dr. Farrell, do you think it possible that your lesions could have sectioned part of that system? It goes right round, and if there was a connection coming back from the rear, this might have been interrupted.

Farrell I doubt it The reticular formation is sectioned at the upper levels of the pons. A certain amount of the reticular structure extends up into the lower thalamic region.

Stack-Dunne There is some suggestion that this system loops forward up into the reticular system and then back again into the

to that, I take it.

Farrell· The mamillary bodies are definitely rostral to the section.

Astwood. Dr. Farrell, would you tell us what are your conclusions from your studies in the dog on control of adrenal function?

stage, we can state that in an animal with a midcollicular section in which the brain tissue rostral to the section has been removed, the rate of aldosterone secretion is quite low; if one administers to this preparation an extract of beef diencephalon, output of aldosterone can be increased to levels higher than normal.

women found no rise in basal metabolic rate (BMR) until the seventh or eighth month, when they did get a small rise.

Pearlman: So that the organism is somewhat protected?

Pitt-Rivers: It is protected against this rise in BMR which is dispersed without producing increased heat; whether the foetus is mopping it up or not we don't know.

Randle: Dr. Astwood, have you done any protein-bound iodine in acromegaly?

Astwood: In acromegaly the protein-bound iodine is usually normal, but occasionally there is thyrotoxicosis in association, and then it is high.

Randle. Can you get raised basal metabolic rate without raised PBI?

Astwood. Yes.

Short: Dr. Bush mentioned the release of corticoids after emotional stress. Dr. Bush, do you consider it to be a hazard of blood transfusion, where the recipient might receive a high level of corticoids from a stressed donor?

Bush: Sandberg and I found that the corticoid concentration in donor blood was normal one month after storage. Other evidence has since confirmed our supposition at the time: under ordinary blood-bank conditions for storage of plasma, survival of the hormones is quite good up to at least one month. I don't think there is any evidence that donor blood is especially rich in corticoids. Most of these donors are quite practised, they come up every two or three months, so many of them are accustomed to the procedure.

Randle: Dr. Farrell, have you tried serotonin on the level of blood ACTH?

Farrell: Dr. Sayers plans to check this but it has not yet been done.

With regard to emotional factors exciting the pituitary adrenal axis, I should like to hear Prof. Harris comment on what he feels [illegible] be to the activation of

[illegible] field. There are some interesting possibilities however, with regard to the hippocampus-amygdaloid complex. As Dr. J. D. Green has shown (Green, J. D., and Arduini, A. A. (1954). *J. Neurophysiol.*, 17, 533), the reticular-activating system projects through the hypothalamus, around to [illegible]

states.

soluble in chloroform. Thus similar differences in plasma level might be due to different causes, and a lack of difference might simply be due to two or more alterations which balance each other. To understand the dynamic relationships of the hormone in various conditions, therefore, means of measuring production, distribution, and removal in the human subject were necessary.

Three groups of workers independently reported the use of intravenous infusion of a standard dose of free cortisol over a short interval as a means of determining rate of metabolism, apparent volume of distribution and, as a derivative of these, production (Brown *et al*, 1954; Peterson *et al.*, 1955; Geyer and Keibl, 1955). All three found that after a short period the decreasing concentrations in peripheral plasma over the next 6–8 hours fell on a logarithmic curve when plotted against time, indicating removal proportionate to concentration. When this curve was extrapolated to zero time, a value representing the concentration if the factors determining the later portion of the curve had not been operating could be obtained. In our own publications this has been called the "apparent distribution volume" (ADV) since presumably the major factor during the early period was the distribution into tissues. Again, all three groups found that, under these conditions, the value in young normal human beings averaged about 90 per cent of the total body mass. Since such a value is higher than the total water, it could not represent simple diffusion in aqueous solution and must, therefore, indicate uneven distribution. From the apparent distribution, however, and the slope of the removal curve, values for production at normal endogenous levels were calculated.

Another development which furnished further information regarding metabolism was the publication of a technique for the determination in plasma of 17-hydroxycorticosteroids (17-OHCS) conjugated with glucuronic acid (Bongiovanni, 1954). Not only could endogenous levels be estimated but, when combined with the cortisol removal test just described, comparison of the time curves of the two values furnished an

EXTRA-ADRENAL FACTORS AFFECTING THE LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN PLASMA*

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Introduction

DURING the last five years several methods have been developed which permit the measurement of normal levels of steroid compounds which have the dihydroxyacetone side-chain, using samples of 30 ml. or less of peripheral blood (Nelson and Samuels, 1952; Silber and Porter, 1954; Peterson *et al.*, 1955; Silber and Busch, 1956). In man and the dog over 80 per cent of the material which can be extracted directly with chloroform from the blood of normal subjects is cortisol (Bush, 1953; Bush and Sandberg, 1953); in abnormal conditions, however, other steroids with the dihydroxyacetone side-chain may be present (Eberlein and Bongiovanni, 1956). By the use of these techniques much has been learned about the variations in blood levels, both during normal existence and in many pathological conditions. The concentration of free 17-hydroxycorticosteroids in the plasma, which is essentially equivalent to that which is chloroform-soluble, is a resultant, however, of production, distribution into the tissues, and metabolism either to compounds without the dihydroxyacetone side-chain or to conjugated compounds no longer

* [illegible] from the [illegible]lical [illegible]ment [illegible]ican [illegible] the National Research Council.

in the disappearance of cortisol from the circulation which increased disproportionately to concentration. Peterson and Wyngaarden (1956) demonstrated that the greater slope in the radioactivity experiments could not be due to the more rapid disappearance of a non-17-hydroxycorticosteroid since they obtained essentially the same half-life for cortisol by plotting specific activities against time as they obtained with total chloroform-soluble radioactivity. Our own studies, discussed later, have shown that both the zero intercept and the slope of the curve of disappearance of chloroform-soluble radioactivity after a tracer dose of cortisol depends on the initial concentration of cortisol.

All of these methods have helped in distinguishing among the effects of various factors on production, distribution or metabolism of cortisol, and in certain instances some more specific mechanism can be inferred. Question can be raised in some instances, as in the determination of conjugated 17-hydroxycorticosteroids, of the completeness of estimation. Since, however, this is not intended to be a conference on methodology, the shortcomings of specific procedures will not be discussed. They are sufficiently quantitative to permit comparison of results obtained by the same method on different groups, even though the values may not be absolute.

The extra-adrenal factors to be considered here are those which primarily affect distribution and disposal, any influence on production being primarily a result of these changes. Specifically, the physiological processes which will be discussed are age, thyroid function, hepatic function, fluid accumulation, and renal function. Their effects on plasma levels of cortisol may be brought about by influences on any of a number of processes which are involved. They may change distribution by altering the forces which determine the equilibrium between intravascular and extravascular concentrations; by affecting the intervening structures and, therefore, the rate at which the hormonal molecules move under a given gradient; or by changing the distribution between cells and extracellular fluid. They may alter disposal by affecting,

estimate of the balance between rate of metabolism and of excretion.

These procedures allowed the estimation of production and metabolism under endogenous adrenal stimulation, but they would not reveal whether alterations in production were due to changes in stimulation or in response. An ACTH infusion test was therefore developed (Eik-Nes *et al.*, 1954*a*). In this test, ACTH was infused intravenously over a six-hour period at a rate which was more than enough to produce maximal stimulation in normal human subjects. Serial samples of blood were drawn during the infusion and the curve with time plotted. The mean curves for different groups were found to fit, within the limits of error, the change expected when there was a constant addition of hormone and removal proportionate to concentration. When the rate of removal found in the cortisol removal test was introduced as the latter value, a rate of addition could be calculated. It was considered that this would be a measure of the maximum ability of the adrenal to respond to the trophic hormone. Christy, Wallace and Jailer (1955) introduced a four-hour ACTH infusion test to measure adrenal function; but, since only the concentrations of hormone in the plasma at the beginning and at the end of the infusion were determined and no cortisol removal curves were run, a similar calculation could not be made.

The synthesis of [4-^{14}C]cortisol made possible the study of cortisol metabolism by radioactive methods. This had the great advantage of allowing measurements without significant alteration in total cortisol levels. As in the case of 17-hydroxycorticosteroids after infusion of exogenous cortisol, the chloroform-soluble radioactivity, after a short period of equilibration, fell on a logarithmic curve. The slopes were greater, however, and the zero intercepts represented dilution equivalent to 15–30 per cent of body volume (Peterson *et al.*, 1955; Migeon *et al.*, 1956*a*). Two explanations of the difference between results of the two procedures were possible; either some chloroform-soluble metabolite not having a dihydroxyacetone side-chain was present or there were factors involved

tively constant high levels in their subjects between 9 a.m. and noon, followed by a fall. We have not found such constant

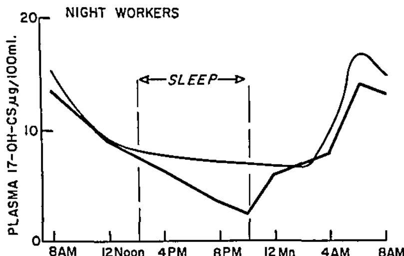


FIG. 1. The mean variation of plasma 17-hydroxycorticosteroids in night workers is shown by the heavy line. Their usual period of sleep is also shown. The light line is derived from a large series of values in normal subjects on a usual schedule of activity and rest.

morning levels; whether this is due to differences in geographic location or to some other factor cannot be judged at this time. The cause of the diurnal variation has not yet been clarified.

Table I

ENDOGENOUS LEVELS OF FREE 17-HYDROXYCORTICOSTEROIDS IN THREE NORMAL YOUNG MEN DURING THE FORENOON

Time	*Ba*	*Ar*	*Ha*
	μg /100 ml.	*μg./100 ml*	*μg./100 ml.*
8 a m.	11 4	17 6	12·6
9 a m.	10 4	15 0	—
10 a. m.	5·4	13 2	10 2
12 noon	5 2	6 8	5 0
2 p m	6 0	7 8	3 4

directly or indirectly, the rate of certain enzymic reactions in the presence of a given substrate concentration; by changing the rate with which substrate reaches the enzyme (alteration in substrate concentration); or by affecting the rate of removal, and therefore the concentration, of product. In most cases we can only infer the process which has been changed.

Effects of cortisol level

Before we can consider variations due to the factors mentioned, it is necessary to know the pattern with which any new condition must be compared, the so-called "normal". As far as can be judged, the steroids which increase in concentration during passage through the human adrenal gland are unconjugated; those released by hydrolysis are as high in the arterial blood as in the adrenal venous blood. In the adrenal venous effluent, where the concentration of cortisol may be as high as 280 µg./100 ml. plasma, both Sweat (personal communication) and Eik-Nes have found that as much as 30 per cent may be in the cellular components. In the peripheral circulation, however, where concentrations are normally between 2–23 µg./100 ml. plasma, the amount present in the cellular layer of a freshly drawn sample is not much greater than that to be expected from occluded plasma. This *difference between blood from the two sources* will be considered later.

While the range of concentration of 17-hydroxycorticosteroids in the plasmas of normal people is wide if no consideration is given to time of sampling, the range of values at any one time of day is considerably less. One of the consistent findings when a good analytical method is used is a diurnal variation (Fig. 1). The curve in our subjects is not symmetrical, the concentration being lowest between 2 and 4 a.m. and then rising quite rapidly between 4 and 8 a.m. The levels then fall at a decreasing rate throughout the day and become fairly steady during the evening hours (Migeon *et al.*, 1956*b*). A series of values on individual young men is shown in Table I. Peterson and Wyngaarden (1956) report that they found rela-

circulation, the ADV, increased with total level (Table II). Apparently, as the concentration of cortisol rises, the proportion which is taken up by the extravascular tissues increases. This is the result to be expected on the basis of the discussion in the last paragraph. The presence of a greater proportion of uncomplexed steroid at higher plasma concentrations is indicated by the greater proportion of chloroform-soluble radioactivity excreted during the first six hours in the urines of those in the above experiment who had higher plasma

Table II

EFFECT OF ORIGINAL CORTISOL LEVEL ON DISTRIBUTION AND RATE OF REMOVAL OF THE HORMONE

Dose and Time	*No Cases*	*Ave. Orig Cortisol Concentration µg /100 ml plasma**	*Slope* $ln \frac{\Delta C}{\Delta t}$	C_o	*ADV***
Tracer, 12 M –6 a.m Ave. Range	4	3 6	– 0 449 0 390–0 502	397 348–488	26 21–29
Tracer, 8 a.m.–2 p.m Ave. Range	9	13 9	– 0 497 0 465–0 550	283 222–348	36 29–45
Cortisol 1 mg /kg., 8 a m.–2 p m. Range	5	126	– 0 317 0 263–0 376	129 118–137	78 73–85

* Concentration at time of injection of tracer
** Apparent distribution volume as per cent total volume.

cortisol levels. When the tracer dose was injected at an average initial level of 3·6 µg./100 ml. plasma, an average of 0·48 per cent of the injected dose was found in this fraction at the end of one hour with a range of 0·29–0·50 per cent among four subjects. When the tracer was given at the beginning of a cortisol removal test, with an average initial level of 126 µg./100 ml. plasma, the average was 1·48 per cent at one hour, with a range among four subjects of 1·38–1·60 per cent. At six hours the respective averages were 1·36 and 3·66 per cent. A greater proportion of the cortisol was ultrafiltrable when the level of cortisol was high. Since a greater proportion of cortisol is diffusible at higher concentrations, it is not surprising that

Early in our studies comparisons were made between the concentrations in various body fluids (Sandberg *et al.*, 1954). Samples of pericardial, pleural and peritoneal fluids were obtained where the major factors responsible for their accumulation were physical rather than inflammatory. Under such conditions the concentrations in the three fluids were always considerably less than in plasma drawn at the same time. The difference could be explained on the basis of the tendency of the adrenal steroids to be held to certain plasma proteins by a combination of electrostatic and van der Waals forces. Since these forces are weak, the complex formed is always in equilibrium with the free steroid. The steroid portion can, therefore, be extracted with organic solvents, the rate of removal during the extraction being the only factor changed by the presence of the protein.

Eik-Nes and co-workers (1954*b*) found that cortisone and cortisol formed weak complexes with serum albumin, and considerably stronger complexes with a mucoprotein separated from plasma. Sandberg, Slaunwhite and Antoniades (1957) report that, while albumin accounts for more of the complexed steroid because of the greater abundance of this protein, fraction IV-1 of Cohn binds more cortisol per unit protein. If some proteins present in small amount bind cortisol more strongly than albumin, these would tend to become saturated first as the concentration of cortisol was increased, and the portion more weakly bound to albumin would represent a greater and greater proportion of the total. Thus the amount of cortisol free to diffuse into extravascular compartments would not be proportionate to concentration, but would increase more rapidly as the concentration rose. Under such conditions the distribution between vascular and extravascular compartments would change with concentration of steroid in the plasma.

As mentioned previously, when the distribution of radioactivity after injection of a tracer dose of [4-^{14}C]cortisol was studied in the presence of various levels of non-radioactive hormone, the proportion of isotopic compound lost from the

processes on distribution and disposal. The values for "apparent distribution volume" (ADV) and rate of metabolism are therefore those associated with initial high levels of cortisol. Since each type of test was run in the same manner throughout the series, however, values between groups are comparable.

In the cortisol removal test each subject was infused with

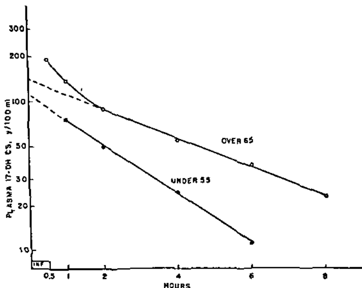


FIG. 3 Mean curves for free 17-hydroxycorticosteroids after a standard cortisol removal test in a series of normal males 23–55 years of age and a series of normal males 66–92 years of age

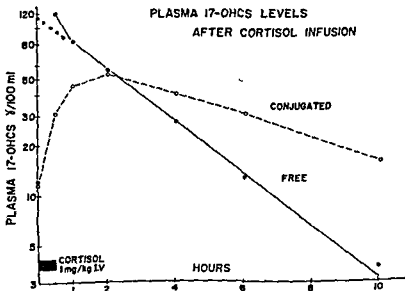
1 mg. cortisol per kg. body weight during a thirty minute period. Samples of blood were drawn before and 30, 60, 120, 240 and 360 minutes after beginning infusion. Fig. 2 gives the average values for free and conjugated 17-hydroxycorticosteroids during such a test in a series of young adults (Brown *et al.*, 1957*b*). In Fig. 3 the curve for free steroids in this group is compared with that of a series of men over 65 years of age. The older men show a slower achievement of equilibrium, indicated by the time which elapsed before the values fell on

a significant proportion might penetrate the cellular components at the concentrations found in adrenal venous blood.

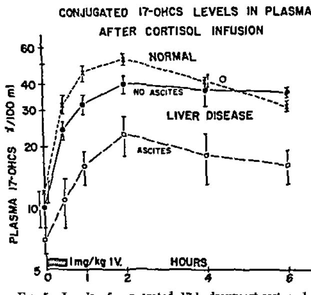
The difference in distribution of the hormone between vascular and extravascular spaces may not be due solely to the plasma proteins, however. The proportion removed from the plasma would be greater with increasing concentration if adsorption occurred on extravascular surfaces, or if certain cells, such as fibroblasts (Dougherty and Schneebeli, 1955), engulfed diffusible cortisol. Perhaps both intravascular and extravascular factors operate to increase the proportion of cortisol leaving the plasma as the circulating level increases. Thus the increase in hormone concentration at the tissue level is greater than the increase in plasma concentration —a very effective system.

Effects of other physiological parameters

The cortisol removal test and the ACTH infusion test have been used to study the influences of the different physiological



corticosteroids (Fig. 5). Not only is the rise more gradual, but the peak is lower and the maintenance of increased levels more prolonged in relation to the concentrations of free cortisol. When there is no ascites, the ADV does not differ significantly from the normal for the same age. Ascites further decreases the slope of the cortisol infusion curve and



increases the ADV because the ascitic fluid serves as an additional reservoir outside the vascular system, and undergoes relatively slow exchange with the plasma (Brown *et al.*, 1954; Klein *et al.*, 1955; Englert *et al.*, 1957*a*).

The ACTH infusion curves of the young and old normal subjects and the patients with hepatic disease are shown in Fig. 6 Judging from these alone, one would assume that the adrenals of the older normal group had the greatest capacity to respond to stimulus, while those of the patients with hepatic

a logarithmic curve, a slower rate of metabolism as measured by the decrease in slope of the curve, and a smaller ADV as shown by the higher intercept at zero time (Samuels, 1956). The slower attainment of equilibrium may reflect the greater density of the extravascular connective tissue. The smaller ADV may also be in part a reflection of the slower penetration

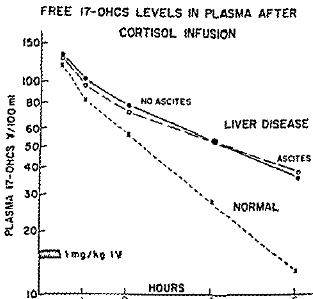


FIG 4 Mean cortisol removal curves after a standard test in normal subjects and patients with liver disease under 55 years of age.

of tissues, in that equilibrium with metabolic disposal may be reached before uniform distribution has been achieved. It is also, probably, a result of the decreased number of fibroblasts in the tissues of the aged. The reduced removal slope must be due to slower metabolism. Since the liver is the major organ involved, it probably represents reduced hepatic activity.

The rate of removal of cortisol is markedly influenced by liver function. If liver function is reduced there is a significant change in the slope of the cortisol removal curve (Fig. 4), and a slower rise in the concentration of conjugated 17-hydroxy-

Composite Change: $\frac{dC}{dt} = k' - kC$

Integrated between C_o and C_x:

$$\ln \frac{\frac{k'}{k} - C_o}{\frac{k'}{k} - C_x} = kt$$

If the rates of removal obtained from the cortisol removal curves are inserted in this equation and the values for rate of production corrected for the ADV, the capacity of the adrenals of both the older group and the patients with hepatic disease is less than that of the organs of the younger normal group. The calculations of normal and maximal production based on these curves are given in Table III. While the average levels at 9 a.m. may be slightly higher in the groups with

Table III

EFFECT OF AGE AND LIVER DAMAGE ON PARAMETERS OF CORTISOL METABOLISM

Subject	*Plasma 17-OHCS 8 a.m.*	*ADV**	*Removal constant*	*Production*	
				Normal	*Maximal (ACTH Test)*
	µg /100 ml	*% B W*	*ln hr⁻¹*	*µg /kg /hr.*	*µg /kg /hr.*
Normals, 23–55 yr , Ave.	13 6	90	– 0 350	21 8	154
Normals, 66–92 yr., Ave	15·6	60	– 0·251	13 7	120
Cirrhosis, 32–54 yr., Ave.	13 5	82	– 0 205	13 3	97

* Apparent Distribution Volume

decreased rates of steroid metabolism, they are within the range for normal young men and must therefore represent some adjustment of production to the decreased removal. Moreover, a chronic decrease in the rate of production appears

disease would appear to be equal to the young normal subjects. As already pointed out, however, these curves appear to fit the condition where a constant increased amount of hormone is

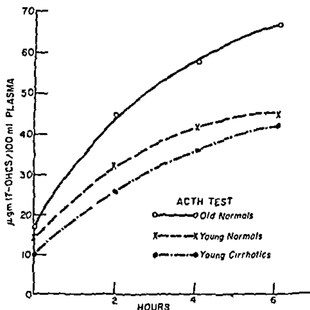


FIG 6. Mean ACTH infusion curves for young and old normal subjects and young subjects with liver damage.

being added and the excess is being removed in proportion to concentration. The integration of such an equation is as follows:

Let C = concentration of 17-hydroxycorticosteroids in μg./100 ml.

t = time

k = slope of cortisol removal curve

k' = constant rate of input

$$\text{Input: } \frac{dC}{dt} = k'$$

$$\text{Removal: } -\frac{dC}{dt} = kC$$

accelerated reduction and conjugation (Fig. 8). There is good reason to think that a significant factor in the postoperative increase in 17-hydroxycorticosteroid values, as well as the

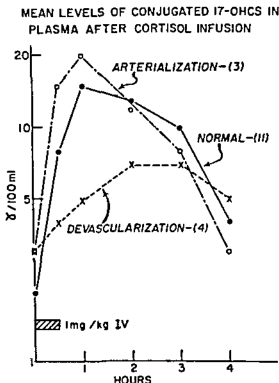


FIG 8 Conjugated 17-hydroxycorticosteroid curves after intravenous infusion of cortisol into dogs with normal or altered hepatic blood flow

premortal rise, is due to reduction in hepatic blood flow (Tyler *et al.*, 1954; Sandberg *et al.*, 1956).

Renal damage influences cortisol metabolism because of its effect on the excretion of the conjugated metabolic products (Englert *et al.*, 1957*b*). As seen in Fig. 9, there is an inverse relationship between creatinine clearance and the endogenous levels of conjugated 17-hydroxycorticosteroids in the plasma.

to lead to some degree of adrenal atrophy so that maximal capacity is decreased.

Not only does cellular damage to the liver affect the rate of removal of cortisol; changes in the rate of blood flow through

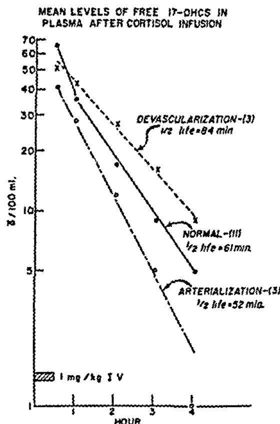


FIG. 7. Rate of removal of intravenously infused cortisol in normal dogs and those with altered hepatic blood flow.

the organ also alter rates of removal and conjugation (Englert *et al.*, 1957c). When the blood flow through the portal system was partially shunted into the vena cava in dogs, the rate of removal of cortisol was considerably reduced; it was increased somewhat by shunting blood from the aorta into the portal vein (Fig. 7). The more rapid removal was associated with

al., 1957*a*), the rate of removal of the hormone was significantly reduced but the ADV was within the normal range (Table IV). In hyperthyroidism, the rate of removal was

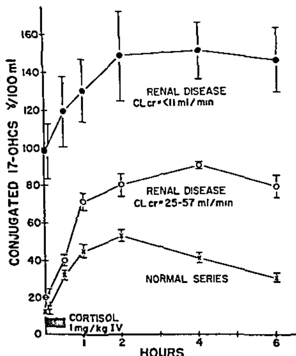


FIG. 10. Mean levels of conjugated 17-hydroxycorticosteroids in plasma of normal subjects, patients with early uraemia, and patients with advanced renal disease, after a standard cortisol removal test

markedly increased while the ADV was significantly decreased. As in the case of the elderly normal subjects, the apparent decrease in distribution volume may be due to the failure of the cortisol to achieve complete equilibrium of distribution before equilibrium with metabolic removal was reached.

When a cortisol removal test is run, there is a marked increase in both height and duration of the elevation in conjugated 17-hydroxycorticosteroids (Fig. 10). This great accumulation of product apparently decreases the rate of the conjugating reactions in the liver since there is a small but significant

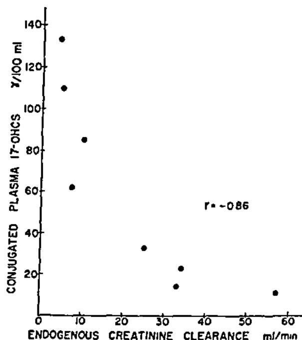


FIG. 9. Scatter diagram depicting correlation between

reduction in the rate of removal of free 17-hydroxycorticosteroids (Fig. 11).

The last extra-adrenal variable to be considered here is thyroid function. Several workers have noted alterations in adrenal metabolism associated with hyper- or hypothyroidism (Engstrom and Mason, 1944; Levin and Daughaday, 1955; Eik-Nes and Brizzee, 1956). When cortisol removal tests were carried out in patients with hypothyroidism (Brown *et*

Table IV

PARAMETERS OF CORTISOL METABOLISM IN A SERIES OF PATIENTS WITH ABNORMAL THYROID FUNCTION

Subject	*BMR* %	*Plasma 17-OHCS 8 a m* μg /100 ml.	*ADV** % B.W	*Removal constant* ln hr.$^{-1}$	*Production* *Normal* μg./kg /hr	*Maximal (ACTH Test)* μg./kg./hr.
Case M, Hypothyroid	− 33		83	− 0 223		
Case P, Hypothyroid	− 20		87	− 0 157		
Case S, Hypothyroid	− 40	17	93	− 0 251	23 2	139
Case S, Overtreated	+ 36	8	70	− 0 661	21 6	183
Case B, Hyperthyroid	+ 66		77	− 0 804		
Case T, Hyperthyroid	+ 33		68	− 0 883		
Case H, Hyperthyroid	+ 42		74	− 0 609		
Case M, Hyperthyroid	+ 34		84	− 0 484		
Hypothyroids, 12 cases, Ave.		13	93	− 0 189	12 7	109
Hyperthyroids, 10 cases, Ave.		11	66	− 0 622	25 3	195

* Apparent Distribution Volume

Here, however, the effect would be due to the speeded-up metabolism instead of to a reduced rate of diffusion. The calculated rates of production indicate decreased adrenal function in hypothyroidism and increased function in hyperthyroidism. The ACTH infusion tests indicate that this is not solely a compensatory increase in an otherwise normal gland,

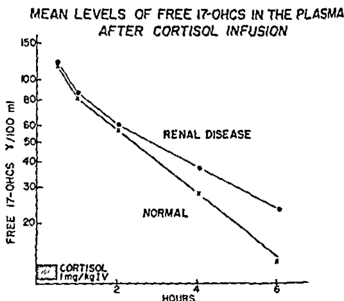


FIG. 11. Mean curves for normal subjects and patients with renal disease in standard cortisol removal test.

but that adrenal capacity is also affected, probably through changes in the metabolic rates of the adrenocortical cells. This change is not so great, however, as in the rate of removal. It seems therefore that thyroid function affects the metabolic activity of both the adrenal and hepatic cells, but that the alterations in the latter are greater as far as the enzymic reactions involving cortisol are concerned. *Whether there is* also a change in the forces [illegible] between blood and tissues in [illegible] whether the effect on ADV [illegible]

Thyroid function is related to cortisol metabolism through its influence on the metabolic rate of both the adrenal and the hepatic cells. The influence on the latter appears to have the greater effect on cortisol turnover. Hyperthyroidism may also alter the distribution between plasma and tissues.

The chemical and radiochemical methods now available provide tools for the study of the dynamics of cortisol metabolism in the human being, and their further application and refinement should provide much more detailed knowledge of the function of the hormone in both normal and pathological conditions.

REFERENCES

BONGIOVANNI, A. M (1954). *J. clin. Endocrin Metab*, **14**, 341.

BROWN, H., ENGLERT, E. Jr., and WALLACH, S. (1957*a*). *J. clin Invest.* Submitted for publication.

BROWN, H, ENGLERT, E. Jr., WALLACH, S., and SIMONS, E. L (1957*b*) *J. clin. Invest* Submitted for publication

BROWN, H, WILLARDSON, D. G., SAMUELS, L. T., and TYLER, F. H. (1954). *J. clin. Invest*, **33**, 1524.

BUSH, I. E. (1953). *J. Endocrin.*, **9**, 95

BUSH, I. E, and SANDBERG, A. A (1953) *J. biol Chem*, **205**, 783.

CHRISTY, N. P., WALLACE, E. Z, and JAILER, J. W. (1955). *J. clin. Invest*, **34**, 899.

DOUGHERTY, T F, and SCHNEEBELI, G. L. (1955). *Ann. N.Y. Acad. Sci.*, **61**, 328

EBERLEIN, W R, and BONGIOVANNI, A. M. (1956). *J. biol Chem*, **223**, 85.

EIK-NES, K, and BRIZZEE, K. R. (1956). *Amer. J. Physiol.*, **184**, 371.

EIK-NES, K, SANDBERG, A A., NELSON, D H, TYLER, F. H., and SAMUELS, L T. (1954*a*). *J. clin. Invest*, **33**, 1502

EIK-NES, K, SCHELLMAN, J. A., LUMRY, R, and SAMUELS, L T. (1954*b*). *J. biol. Chem.*, **206**, 411.

ENGLERT, E Jr, BROWN, H, WALLACH, S, and SIMONS, E L. (1957*a*). *J clin Invest.* Submitted for publication.

ENGLERT, E Jr., BROWN, H, WILLARDSON, D. G, WALLACH, S., and SIMONS, E. L. (1957*b*) *J. clin Invest.* Submitted for publication.

ENGLERT, E. Jr., NELSON, R M, BROWN, H., NIELSEN, T. W., and CHOU, S. N. (1957*a*). [illegible]

of cortisol by the liver remains to be determined by more critical means. In any case, the great increase in rate of conjugation and excretion must affect the relationship between changes in secretion by the adrenal gland and alterations in effective concentration in the peripheral tissues.

Summary

The determination of the endogenous levels of free 17-hydroxycorticosteroids in plasma, largely cortisol in the human being, is useful; it fails, however, to reveal many differences in adrenal function because of the compensating mechanisms which are involved. Methods utilizing measurements on serial samples of blood to estimate both distribution of these substances between blood and tissues and their metabolic disposal have been developed.

The level of cortisol in the plasma affects the proportionate distribution between blood and tissues. As concentration increases the proportion of freely diffusible cortisol apparently increases. In addition, the proportion which is removed by adsorption on surfaces or by engulfment by cells probably increases. The net effect is to make the increased level of the hormone more available proportionately to tissues.

Age also affects cortisol metabolism. Beyond 65 years of age there is a significant decrease in the rate and volume of distribution to the tissues, and in the rate of metabolic removal.

Hepatic damage primarily reduces the rate of metabolic disposal. In the presence of an undamaged liver, however, alterations in hepatic blood flow will also change the rate of cortisol metabolism.

Renal damage indirectly affects the rate at which free cortisol is removed from the circulation through accumulation of the conjugated end-products. There is a direct relationship between the clearance of conjugated 17-hydroxycorticosteroids and creatinine clearance, indicating that glomerular filtration is a major factor in disposal of the steroid metabolites.

Thyroid function is related to cortisol metabolism through its influence on the metabolic rate of both the adrenal and the hepatic cells. The influence on the latter appears to have the greater effect on cortisol turnover. Hyperthyroidism may also alter the distribution between plasma and tissues.

The chemical and radiochemical methods now available provide tools for the study of the dynamics of cortisol metabolism in the human being, and their further application and refinement should provide much more detailed knowledge of the function of the hormone in both normal and pathological conditions.

REFERENCES

BONGIOVANNI, A. M. (1954). *J. clin. Endocrin. Metab*, 14, 341.

BROWN, H., ENGLERT, E. Jr, and WALLACH, S. (1957a). *J. clin. Invest.* Submitted for publication.

BROWN, H, ENGLERT, E. Jr, WALLACH, S, and SIMONS, E. L. (1957b). *J. clin. Invest* Submitted for publication.

BROWN, H., WILLARDSON, D G, SAMUELS, L. T., and TYLER, F H. (1954). *J. clin Invest*, 33, 1524.

BUSH, I. E. (1953) *J. Endocrin*, 9, 95

BUSH, I. E, and SANDBERG, A. A. (1953). *J. biol. Chem.*, 205, 783

CHRISTY, N. P, WALLACE, E Z., and JAILER, J. W (1955) *J. clin. Invest*, 34, 899.

DOUGHERTY, T. F., and SCHNEEBELI, G L (1955). *Ann. N.Y. Acad. Sci.*, 61, 328.

EBERLEIN, W. R, and BONGIOVANNI, A. M. (1956). *J. biol. Chem.*, 223, 85.

EIK-NES, K., and BRIZZEE, K. R. (1956) *Amer J. Physiol*, 184, 371.

EIK-NES, K, SANDBERG, A. A, NELSON, D. H, TYLER, F. H., and SAMUELS, L. T. (1954a). *J. clin. Invest*, 33, 1502

EIK-NES, K., SCHELLMAN, J. A., LUMRY, R., and SAMUELS, L. T. (1954b). *J. biol Chem*, 206, 411.

ENGLERT, E. Jr, BROWN, H, WALLACH, S., and SIMONS, E L (1957a). *J clin Invest.* Submitted for publication.

ENGLERT, E. Jr., BROWN, H, WILLARDSON, D G., WALLACH, S., and SIMONS, E. L. (1957b) *J clin Invest.* Submitted for publication.

ENGLERT, E. Jr, NELSON, R. M., BROWN, H, NIELSEN, T W., and CHOU, S N (1957c) *Surg. Forum.* In press.

ENGSTROM, W. W., and MASON, H. L (1944). *J. clin Endocrin.*, 4, 517.

MIGEON, C. J., SANDBERG, A. A., DECKER, H. A., SMITH, D. F., PAUL, A. C., and SAMUELS, L T. (1956*a*). *J. clin. Endocrin. Metab.*, 16, 1137.

MIGEON, C. J., TYLER, F. H, MAHONEY, J. P., FLORENTIN, A. A., CASTLE, H., BLISS, E. L., and SAMUELS, L. T. (1956*b*). *J. clin. Endocrin. Metab*, 16, 622.

NELSON, D. H., and SAMUELS, L. T. (1952). *J. clin. Endocrin. Metab.*, 12, 519.

PETERSON, R. E., and WYNGAARDEN, J. B. (1956). *J. clin. Invest.*, 35, 552.

PETERSON, R. E., WYNGAARDEN, J. B., GUERRA, S. L., BRODIE, B B, and BUNIM, J. J. (1955). *J. clin. Invest.*, 34, 1779.

SAMUELS, L. T. (1956). Hormones and the Aging Process, p. 21. New York, Academic Press.

SANDBERG, A. A, EIK-NES, K., MIGEON, C. J., and SAMUELS, L.T. (1956). *J. clin. Endocrin. Metab.*, 16, 1001.

SANDBERG, A. A., EIK-NES, K., NELSON, D. H., and TYLER, F. H. (1954). *J. Lab. clin. Med.*, 43, 874.

SANDBERG, A. A., SLAUNWHITE, W. R. Jr., and ANTONIADES, H N. (1957). *Recent Progr Hormone Res*, 13. In press.

SILBER, R. H., and BUSCH, R. D. (1956). *J. clin. Endocrin. Metab.*, 16, 1333.

SILBER, R. H., and PORTER, C. C. (1954). *J. biol. Chem.*, 210, 923.

TYLER, F. H, SCHMIDT, C. D., EIK-NES, K., BROWN, H., and SAMUELS, L. T. (1954). *J. clin. Invest*, 33, 1517.

DISCUSSION

Heller: Have you combined your distribution studies with estimations [illegible]

[illegible] ned by [illegible] nd Dr. [illegible] ing up [illegible] healthy [illegible] amples of fat, abdominal muscles and liver were taken The amount injected [illegible]

[illegible] actually the fat level was if anything a little under what might have been explained by extra-cellular space in the fatty tissues, though it probably could be within that range.

excluded begin to penetrate. If you protect the metabolism of the red cell you reduce or prevent this. Then, if you have an inflammation of the central nervous system, the concentration in cerebrospinal fluid, which is extremely low normally, increases markedly. This is also the time when such things as iodine will now pass the blood-brain barrier. So I have in the back of my mind an idea to be further explored, the idea that the same systems which actively prevent the entry of other

[illegible]

radioactive steroid. Those studies, of course, were made where concen-

[illegible]

the red cells?

[illegible]

state was reached [illegible]

few days

[illegible]

significant increase in rate of production So our interpretation of these

two factors has been that the thyroid hormone increases the total energ
supply to the enzyme systems, both in the liver and in the adrenal, an
that the hepatic increase is a greater one than the adrenal, proportion
ately; this may be simply because of the greater mass. If the destructio
were greater one would anticipate that you should get a slight lowerin
of levels, and that doesn't seem to be true. So perhaps the appare
difference between production and destruction is one that is artificial i
the experiment, but actually they are in balance in the individual.

Prunty. I think there is a balance because, if you study the end orga
effects in respect to blood sugar and sodium retention under these con
ditions, they both increase. This suggests that it is not merely a quic
removal of more rapidly metabolized hormone.

Samuels: This raises another interesting question; if we have an in
crease in the metabolic rate of cells in the target organs, can it not b
that the effect of a given amount of the hormone may be increased?

Prunty: Yes, it could be.

Bush: Prof. Prunty, did your slide refer to a constant administratio
of cortisol, or were the figures there of *endogenous* steroid productio
under ACTH stimulation?

Prunty. There was constant administration of ACTH.

Bush. Dr. Samuels, you gave some figures on the excretion of freely
extractable counts per minute in your tracer experiments. Did I gathe
correctly that your first sample was obtained during a cortisol infusion
in both experiments in which you compared the two?

Samuels: No.

Bush: I see, the first one was an F infusion, and you had a plasma
value of 108 µg./100 ml

In the other experiments where your urinary-free F, or counts pe
minute per litre was about one-third of the previous experiment, and the
plasma value was 3 0, you were in fact attributing the difference to the
plasma concentration rather than to the time at which the experiment
was done?

Samuels: The tracer was given as a single immediate injection in both
cases. The tracer would be more dilute in the case where the level was
108 µg./100 ml. plasma, so that if the same proportion of the level was
removed, in other words if you were filtering out thirty times as much,
or twenty-five times as much in one case as you were in the other, you
would still get only the same amount of isotope out. When you get a
greater proportion of isotope excreted, you must have had an increase,
not only proportionate to concentration, but even greater. Therefore,
the ratio between unfiltrable and bound hormone must have increased

Bush: I think I can show a graph this afternoon that might show this
explanation to be possible.

CIRCULATING STEROID HORMONE LEVELS IN RELATION TO STEROID HORMONE PRODUCTION

WILLIAM H. PEARLMAN
Guy's Hospital Medical School, London

The Turnover Time of Endogenous Hormone in the Blood Pool

Introduction

IN the following presentation, an effort will be made to establish a quantitative relationship between the amount of steroid hormone which the body produces and the amount which circulates in the blood stream. At the steady state when the endogenous production rate (r) and the total amount (M) of circulating hormone are constant, the following relationship obtains:

$$\frac{M}{r} = T \qquad \ldots. (1)$$

The symbols M and r have already been defined; T is the turnover time, i e. the time required for the complete replacement of the circulating hormone by a fresh supply from the endocrine gland. Since M is a product of the blood hormone concentration (C) and the blood volume (V), we have:

$$T = \frac{CV}{r} \qquad \ldots. (2)$$

Thus the blood hormone level is a function of the product of the hormone production rate (r) and the turnover time (T) in the blood pool. As the response of the target organs is dependent on the blood hormone concentration, it is important to understand not only the factors controlling the

turnover time in the blood pool under normal and pathological conditions.

In connection with equation (2), it is desirable to know, at the moment the blood sample is withdrawn for hormone estimation, the instantaneous rate of endogenous hormone production rather than the average rate over a long period of time. The following procedures have been employed in estimating hormone production rate:

(*a*) A known amount of the hormone is injected and the percentage urinary recovery as the original hormone or its metabolites is determined. From the urinary steroid excretion rate in the control or pre-injection period, one may then estimate the average rate of endogenous hormone production This estimate is subject to certain reservations since it is assumed that the rate of hormone metabolism is uniform throughout the post-injection collection period and is identical with that in the pre-injection control period. It should also be noted that the dose of hormone administered usually exceeds the physiological level.

(*b*) A more reliable and accurate estimate of the average rate of hormone production may be obtained by a procedure similar to that above but employing isotopically labelled hormone. One need determine only the specific activity of the urinary excretion product since the amount and specific activity of the hormone injected are known. Most of the assumptions made in applying procedure (*a*) above apply here as well, although a tracer dose of hormone may be administered. However, the analytical errors are considerably reduced. It is assumed that the urinary metabolite is derived entirely from the hormone, which may not necessarily be the case.

(*c*) The instantaneous rate of hormone secretion may be calculated from the difference in the arterio-venous hormone concentrations of the endocrine organ in question and the rate of flow of blood through that organ. Experimentally, this procedure is difficult to perform and is of limited application. It may also be subject to serious technical inaccuracies.

(*d*) The instantaneous hormone production rate may also be determined by injecting a trace amount of isotopically labelled hormone and observing the rate of decline of the specific activity of the hormone in serial blood samples obtained at stated intervals.

The last mentioned procedure requires further description (for the theory of tracer methods, see SIRI, 1949). The amount by weight of hormone injected must be very small in relation to the total amount of inert hormone circulating if the system is not to be disturbed; a steady state must be maintained in which the hormone blood level and the rate of endogenous hormone production remain constant. A first order reaction may be expected wherein the rate of declining concentration of radioactive hormone is directly proportional to its concentration at any given instant:

$$\frac{dC}{dt} = -kC \qquad \ldots (3)$$

The integrated equation is:

$$C = C_o e^{-kt} \qquad \ldots. (4)$$

or

$$2.3 \log \frac{C_o}{C} = kt$$

where C_o is the initial concentration of radioactive hormone and C the concentration after t minutes, and k is a constant called the turnover rate. The value for k is obtained most readily when $C = 0 \cdot 5C_o$ for then equation (4) becomes:

$$k = \frac{0 \cdot 693}{t_{\frac{1}{2}}} \qquad \ldots. (5)$$

in which $t_{\frac{1}{2}}$ is designated as the half-life time. The turnover rate, k, is the fraction $\frac{r}{M}$ of the total amount of hormone replaced per unit time. M is the total amount of hormone (labelled and unlabelled); r is the actual rate at which the hormone leaves the vascular compartment and hence, at the steady state, is the rate of production of endogenous hormone.

The reciprocal of the turnover rate (k) is called the turnover time (T).

The procedures just described have been employed by various investigators. The data thus obtained may be utilized in estimating turnover time, as will be discussed below. The turnover time values thus far derived are approximate in most cases.

Progesterone

The rate of production of endogenous progesterone in late pregnancy was determined by Pearlman (1957) to be of the order of 0·25 g. per day; the calculations were based on isotope dilution procedure involving the administration of [16-^{3}H] progesterone and the subsequent isolation and determination of the specific activity of urinary pregnanediol. Zander and Munstermann (1956) have independently arrived at a similar conclusion. They estimated an endogenous progesterone production of 190 to 280 mg. per day towards the end of pregnancy on the basis of the progesterone content of uterine vein blood (Zander and Münstermann, 1954) and the uterine blood flow. Hence, about 170 μg. progesterone enters and leaves the vascular compartment per minute in late pregnancy. This is an average value taken over the course of 24 hours; it may vary appreciably from one minute to the next and also from one individual to another at any given stage of pregnancy. According to Zander (1955), the progesterone level in late pregnancy is 0·08 μg. per ml. blood. This value, likewise, is an average one, the range being 0·02 to 0·15 μg./ml. blood. These determinations were based on paper chromatography of blood extracts and measurement of the optical density at 240 mμ. of the eluates of the progesterone "spot"; the final identification of the hormone was made by infrared absorption analysis (Zander and Simmer, 1954). Now, if the total blood volume in pregnancy is assumed to be about 7000 ml. and the above values are substituted in equation (2), we arrive at a turnover time of progesterone of about 3·3 minutes. In this short interval, the total amount of progesterone in circulation

has been replaced by a fresh supply, chiefly from the placenta. This is a crude approximation, but one which is none the less quite instructive. For example, one would expect the blood progesterone level to fall precipitously to non-pregnancy levels within a half-hour or less following parturition. At present, direct chemical estimation of the low progesterone blood level in non-pregnancy is exceedingly difficult for lack of a sufficiently sensitive and specific method; an indirect and approximate estimate might be made by application of equation (2) after having determined the rate of endogenous progesterone production. It has not been established, however, whether the turnover time varies with the endocrine status of the individual. In this connection, it is pertinent that the intermediary metabolism of [16-^{3}H]progesterone in pregnancy was found not to differ markedly from that in non-pregnancy in the absence of luteal function (Pearlman, 1957). The validity of the tritium label for this purpose was established in a study in collaboration with Dr. Tait and others (to be published). A solution of [16-^{3}H]progesterone and [4-^{14}C]progesterone was injected into a cancer patient; the ratio of ^{3}H/^{14}C in the urinary pregnanediol was found to be identical, within the limits of experimental error, with that of the hormone injected.

The most important factor accounting for the rapid disappearance of progesterone from the blood stream in pregnancy is probably the rapid rate at which the hormone undergoes metabolic transformation into biologically inactive products. This may be inferred from the following considerations: (*a*) very little of the hormone as such accumulates in the body, and (*b*) the amount of progesterone in pregnancy urine is a very small fraction of the total amount produced in the body in marked contrast with the prodigious quantities of the urinary metabolites. That the liver is an important site of progesterone metabolism has been demonstrated in experiments on animals. In a case of infective hepatitis in a woman in the 16th week of pregnancy, the progesterone blood level was found to be very high, about 0·4 μg./ml. blood (Butt,

Morris, Morris and Williams, 1951). Since endogenous hormone production in early pregnancy is low, one suspects that the turnover time of blood progesterone may have been very much prolonged in this case.

Oestrogens

The turnover time of oestrogens in the blood in pregnancy may be derived in a fashion similar to that just described for progesterone. Pearlman, Pearlman and Rakoff (1954) injected [6 : 7-^{2}H]oestrone acetate into women in late pregnancy and determined the recovery of the isotope in the major urinary oestrogens; the course of oestrone metabolism was found not to differ appreciably from that in non-pregnancy, and presumably this applies also to oestradiol-17β and oestriol. Accordingly, one may estimate that about 85, 5 and 20 mg. oestriol, oestradiol-17β and oestrone, respectively, are elaborated per day in late pregnancy; the oestrone figure is based on isotope dilution procedure, the others on the recovery of urinary oestrogen following the administration of the inert hormones in non-pregnancy. Now, as to the blood oestrogen concentrations in human pregnancy; these are difficult to express in terms of microgram amounts of the individual oestrogens from the bioassay data available in the literature. Goldberger and Frank (1942) found 600 to 1300 mouse units of oestrogenic activity per l. blood in late pregnancy, employing alcohol-ether extracts of blood. Rakoff, Paschkis and Cantarow (1943) found 80 to 120 mouse units of free oestrogen per 100 ml. serum in late pregnancy, obtained by ether-alcohol extraction; the concentration of oestrogens in the serum was identical with that of whole blood. According to Szego and Roberts (1946), the major oestrogenic activity in human pregnancy blood is associated with a fraction identical with or resembling oestriol; in advanced pregnancy (5·5 months) a dialysate of blood contained by bioassay the equivalent of 0·55 μg. oestradiol-17β per 100 ml. blood. Diczfalusy (1953) obtained blood from patients immediately following delivery of the placenta and found by fluorimetry

41·8, 1·6 and 1·9 µg. free or unconjugated oestriol, oestrone and oestradiol-17β, respectively, per kg. blood. From Diczfalusy's data and those cited above on endogenous oestrogen production, the turnover time of the various oestrogens in blood may be calculated to be about 6 minutes or less; considerably more data, preferably on the same experimental subject, are needed for a better approximation. Like progesterone, the oestrogens have a short turnover time in the blood stream, the liver being an important site of metabolism and biological inactivation. By way of contrast, it is noteworthy that Zondek and Black (1947) observed normal blood (and urine) levels of endogenous oestrogen in severe cases of infective hepatitis in pregnancy. These investigators suggest that the acutely diseased liver in an advanced stage of atrophy is still able to inactivate the large amounts of endogenous oestrogens produced during pregnancy.

Testosterone

There is not at present sufficient data to calculate the turnover time of endogenous testosterone, but it is probably quite short. Fukushima, Bradlow, Dobriner and Gallagher (1954) determined, in experiments with isotopic testosterone, that the upper limit of testosterone production in a normal young man was about 17 mg. per day. McCullagh and Osborn (1938) prepared an extract of 50 ml. unhydrolysed blood (obtained from a young man) and observed that it gave no capon comb growth; acid hydrolysis gave 4 i.u. per 100 ml. blood. During a discussion at a recent Laurentian Hormone Conference, Migeon (1956*a*) mentioned that he was unable to detect testosterone in as much as 2 5 l. human plasma. This suggests that the turnover time of testosterone in the blood pool must be very short. Slaunwhite and Sandberg (1956) recently reported in an abstract on the rate of disappearance of intravenously injected [4-^{14}C]testosterone in human subjects. The radioactivity of the unconjugated steroids in the plasma disappeared exponentially at two rates with half-lives of 10 and 75 minutes. The radioactivity contributed by

plasma testosterone was apparently not determined, and consequently one cannot state precisely its rate of disappearance nor derive a turnover time value for the hormone in the blood pool. The precise nature and amount of the androgens secreted in the human species is not well established, nor that of the active (free) androgens in the blood, although data on conjugated C_{19} steroids in the blood have been accruing in recent years (for example, Migeon, 1956*b*).

17-Hydroxycorticoids (cortisone plus cortisol)

From metabolism experiments with [4-^{14}C]cortisol, Hellman and co-workers (1954) have estimated that the human adrenal supplies about 30 mg. cortisol or similar steroids per day. From the data furnished by Morris and Williams (1953), the mean 17-hydroxycorticoid content (cortisone plus cortisol) in normal subjects is $0 \cdot 123\ \mu$g. per ml. plasma. From the two sets of data and by application of equation (2) above, assuming a total plasma volume of 3·3 l. and that 75 per cent of the blood corticosteroid is in the plasma, the turnover time of endogenous 17-hydroxycorticoids in the blood is calculated to be about 26 minutes. This value is by comparison much longer than that for progesterone (3·3 minutes).

Another approach to an approximate estimate of the turnover time may be made on the basis of the data furnished by Bondy and Altrock (1953). These investigators determined the rate of secretion of 17-hydroxycorticoids in normal human subjects by collecting simultaneously samples of left renal vein and arterial blood and determining the difference in concentration of the hormone; from the renal blood flow rate, the rate of endogenous production of 17-hydroxycorticoids was calculated. Utilizing their data, this author derived a turnover time of 31, 26, 73 and 19 minutes in the four normal subjects respectively; the mean value is thus 37 minutes.

Peterson and Wyngaarden (1956) have determined by tracer methods the turnover rate of cortisol in man and its miscible pool. The amount of [4-^{14}C]cortisol which was intravenously injected was sufficiently small, for example 200–500

μg., to satisfy the tracer requirement; in the serial plasma extracts, the labelled hormone was isolated by paper chromatography and its specific activity determined. The logarithm of the specific activity of the plasma hormone was plotted against time. There was initially a rapid decline lasting about 20 to 30 minutes, which was regarded as a period of equilibration of the labelled steroid in an exchangeable cortisol pool. In the subsequent period, the curve was linear; extrapolation to zero time gave the specific activity of the cortisol in the miscible pool. The half-life time of this pool was found to be 83 minutes (average) and the rate of endogenous hormone production ranged from 17 to 29 mg. per day (nine normal subjects). The volume of the miscible pool was obtained by dividing the amount of hormone in this pool by the plasma hormone concentration. The apparent volume of the miscible pool was thus found to range from 8 to 17 l. As the authors have pointed out, "it is unlikely that the volume has any physical reality, since protein binding in the extravascular spaces is probably not quantitatively identical with that in plasma. . . ." From Peterson and Wyngaarden's data, no definite conclusion can be made as to the turnover rate of plasma cortisol, nor as a matter of fact do the authors venture to do so. However, the following interpretation of the experimental data might be made. The radioactive hormone is uniformly distributed within the plasma pool almost immediately following injection. The plasma hormone is fairly rapidly metabolized as indicated by the steep decline in its specific activity during the initial period (20 to 30 minutes). Simultaneously, the labelled plasma hormone slowly equilibrates with an unlabelled pre-existing hormone pool, for example in the endocrine gland, liver, and possibly elsewhere. In the subsequent period, the latter pool which has now attained a maximal specific activity, slowly equilibrates with the plasma hormone pool which now possesses a low specific activity. In consequence, the specific activity of the plasma cortisol appears to decline slowly. How accurately are these processes reflected by the data on exponential rates? The

difficulties in interpretation are similar in certain respects with those encountered by Hellman, Rosenfeld and Gallagher (1954) with regard to the turnover rate of plasma cholesterol. However, if one substitutes in equation (2) above Peterson and Wyngaarden's data on plasma hormone concentration and endogenous hormone production rate, assuming that 75 per cent of the blood cortisol is in the plasma and that the plasma volume is 55 per cent of the total blood volume (6 l.), the apparent turnover time of cortisol in the blood pool may be calculated to be 47 minutes (range 21 to 90 minutes in nine normal subjects). In a similar study of [4-^{14}C]cortisol metabolism in man by Migeon and co-workers (1956), only the plasma "free" radioactivity (chloroform extractable) was determined and not that of cortisol. Consequently, it is not possible to state precisely the rate of disappearance of the hormone

Aldosterone and Corticosterone

Studies on [16-^{3}H]aldosterone and corticosterone will be discussed in the presentation by Dr. Tait.

The Half-Life Time of Exogenous Steroid Hormones in the Peripheral Circulation

Introduction

Inasmuch as the rate of hormone metabolism is probably the most important factor affecting not only the turnover time but also the rate of disappearance of exogenous hormone from the blood stream, a brief review of studies of the latter type is pertinent. Although a parallel may be expected between the two sets of data, one must bear in mind that the half-life time of exogenous hormone in the blood cannot be employed in calculating the turnover time, as these studies come under different categories. In many of the experiments about to be described, the initial level of exogenous hormone in the blood far exceeds the physiological level, particularly in the case of progesterone and testosterone.

The investigators usually plot the logarithm of the blood hormone concentration against time; a straight line plot is observed, suggesting a first order reaction. Actually, there appear to be two or possibly more first order reactions: initially, the slope (k) of the curve in the semi-logarithmic plot is steep and of short duration, and may be referred to as the minor component; it is noteworthy that the slope of the major component appears to remain constant as physiological hormone levels are approached. The half-life time ($t_{\frac{1}{2}}$) of the respective components may be obtained from the equation $k = \frac{0 \cdot 693}{t_{\frac{1}{2}}}$. The various steroid hormones will be discussed and the half-life time of the major component will be the one given.

Progesterone

In the pioneer experiments of Haskins (1950), about 10 mg. progesterone were injected intravenously into each rabbit and the hormone concentration estimated by determining the optical density at 240 mμ. of extracts of plasma samples subsequently obtained at stated intervals. Later, Zarrow, Shoger and Lazo-Wasem (1954) carried out similar experiments, except that progesterone was estimated by bioassay employing the intra-uterine technique of Hooker and Forbes (1949). Haskins (1950) and also Zarrow, Shoger and Lazo-Wasem (1954) plotted the hormone concentration against time; if one recasts their data as a semi-logarithmic plot, a linear relationship with time will be observed. The data furnished by the two groups of investigators were found in this fashion to be in good agreement. The minor component of the curve is of very short duration and is ill-defined. The half-life time (major component) is 4·9 and 11·5 minutes in intact and hepatectomized rabbits respectively, based on the data of Haskins (1950); one obtains similarly 3·3 and 9·1 minutes respectively from the data of Zarrow, Shoger and Lazo-Wasem (1954). It is significant that, in these experiments in which the dose of administered progesterone is large,

there occurs during the initial period a rapid diffusion of the hormone from the circulation into the tissues, particularly into the fat depots (Zarrow, Shoger and Lazo-Wazem, 1954); however, within an hour, the hormone is no longer detectable in the fat. Of pertinent interest is the observation by Kaufmann and Zander (1956) that there is about 1·7 μg. endogenous progesterone per g. fat tissue in women at the end of pregnancy. In animal experiments in which trace amounts of [21-^{14}C]progesterone were administered (Barry *et al.*, 1952), the hormone did not accumulate to any significant extent in any of the organs or tissues examined.

In experimental studies on man, the amount of progesterone intravenously administered was likewise far above the physiological level; the sampling of blood was too infrequent to permit a precise determination of the rate of disappearance of the hormone. Thus, Haskins (1950) injected 100 mg. progesterone intravenously into a pregnant woman with antepartum eclampsia and found a progesterone concentration of 3·8 and 1·6 μg./ml. plasma after 1 and 8 minutes respectively. Zander (1954) injected 200 mg. progesterone intravenously into menopausal or ovariectomized women; the hormone concentrations were 1·44, 0·116 and $<0\cdot1$ μg./ml blood after 3–5 minutes, 2 hours and 24 hours respectively. These results clearly indicate that exogenous progesterone rapidly disappears from the blood stream in man.

Testosterone

Haskins (1950) observed the rate of disappearance of intravenously injected testosterone in rabbits, employing the same procedures and analytical techniques as in the case of progesterone (see above). His data, when replotted as the logarithm of the blood hormone concentration against time, suggest a linear relationship; the half-life time (major component) is 8·2 and 9·3 minutes in the intact and hepatectomized rabbit respectively. Thus, hepatectomy does not appear to influence markedly the rate of disappearance of the hormone. However, hepatectomy-nephrectomy markedly prolongs the testo-

sterone level according to West (1951), from whose data one may derive a half-life time of 4·1 and 46 minutes in the intact and hepatectomized - nephrectomized rabbit respectively. There is an initial period in which the testosterone is found in high concentration in the fat stores. West, Tyler, Brown and Samuels (1951), also West, Tyler and Brown (1951), infused testosterone intravenously into human subjects in doses of 147 to 186 mg. Blood samples were taken at intervals and testosterone determined by measuring the absorption of the extracts at 240 mμ. The investigators clearly demonstrated that, following a short equilibration period, the testosterone concentration in the blood declined in a linear logarithmic course with respect to time. From their data, one obtains a half-life time (major component) of 17 minutes. The rate of disappearance of testosterone in patients with liver disease (cirrhosis) did not appear to differ significantly from that in normal subjects.

Oestrogens

It is well known that oestrogens are rapidly metabolized in the body and that the liver is a site of major importance. There are, however, no extensive data to indicate the precise rate of disappearance of exogenous oestrogen from the blood stream. Rakoff and co-workers (1944) injected a dog with 250,000 i.u. oestradiol intravenously and found 1200, 800 and 530 i.u./100 ml. serum after 10 minutes, 1 hour and 5 hours respectively. It would seem that there is initially a very rapid disappearance of oestrogen but that the small amount of oestrogen which remains disappears slowly. It will be noted that the dosage was much above the physiological range and that the oestrogens in the blood were not partitioned before bioassay; undoubtedly metabolites such as oestrone must have been formed.

Cortisol

The following studies show that exogenous 17-hydroxy-corticoids disappear from the blood stream slowly, that is by

comparison with progesterone. It may be recalled that a similar relationship was obtained as regards the turnover time of the respective endogenous hormones.

Peterson and collaborators (1955) infused 50 to 100 mg. amounts of cortisol intravenously over a period of 20 to 30 minutes in human subjects. They plotted the logarithm of the plasma cortisol concentration against time; a linear relationship was obtained, and the half-life (major component) was 114 ± 6 5 minutes. The determination of cortisol was based not on the isolation of this compound but on the optical density at 410 mμ. after treatment of the plasma extracts with phenylhydrazine; however, cortisol is largely measured thereby. Peterson and co-workers (1955) also infused trace amounts of [4-^{14}C]cortisol and observed a half-life time of 60 to 90 minutes, based on the amount of isotope in the plasma. As they have mentioned, this does not represent unaltered cortisol, and, indeed, a large fraction is not cortisol. In a similar experiment, Hellman and co-workers (1954) found that a maximum of 13 per cent of the circulating radioactivity was present as unaltered cortisol 15 minutes after the end of the infusion. Sayers and collaborators (1955) infused 100 mg. cortisol intravenously over a period of 20 to 40 minutes. Plasma cortisol was determined by silica gel chromatography and fluorescence; the half-life time of the hormone was 80 to 140 minutes.

The liver is a very important factor in determining the rate of disappearance of exogenous cortisol. In the infusion experiment of Peterson and co-workers (1955), the plasma levels of this compound remained much higher in patients with cirrhosis. The same observation was made in an earlier study of a similar nature by Brown and collaborators (1954). Harding and Nelson (1952) had observed a marked difference in the 17-hydroxycorticoid levels in arterial and hepatic venous blood of a dog during and after infusion with cortisone.

That the liver plays a central rôle in the metabolism of steroid hormones was amply indicated by Hellman and collaborators (1956) in tracer studies of the absorption and meta-

bolic fate of [4-^{14}C]cortisol, cortisone, corticosterone and testosterone.

Concluding Remarks

The turnover time of steroid hormone in the blood pool expresses the quantitative relationship between the amount of hormone produced and the circulating blood hormone level. It appears to be chiefly an index of the rate of metabolism of the hormone, the liver being the major site of this process. The turnover time indicates a net effect but does not reveal the nature of the metabolic process. The turnover time may be readily calculated by estimating the blood hormone level and the hormone production rate; the latter is most simply obtained by method (b) (cited above) which requires but a single determination, that of the specific activity of a urinary hormone product. Hormones such as progesterone and the oestrogens have a very short turnover time in the blood, for example about 3 to 6 minutes, in contrast to that of cortisol which is about 26 to 47 minutes.

REFERENCES

BARRY, M. C, EIDINOFF, M. L., DOBRINER, K, and GALLAGHER, T. F. (1952) *Endocrinology*, 50, 587.

BONDY, P. K, and ALTROCK, J R (1953). *J clin Invest.*, 32, 703.

BROWN, H, WILLARDSON, D. G, SAMUELS, L. T, and TYLER, F H (1954). *J clin. Invest*, 33, 1524

BUTT, W R, MORRIS, P, MORRIS, C J. O. R, and WILLIAMS, D. C. (1951). *Biochem J.*, 49, 434.

DICZFALUSY, E (1953). *Acta endocr., Copenhagen*, Supp. No. 12.

FUKUSHIMA, D K, BRADLOW, H. L, DOBRINER, K, and GALLAGHER, T F (1954) *J. biol Chem*, 206, 863.

GOLDBERGER, M A., and FRANK, R. T. (1942) *Amer. J Obstet. Gynec*, 43, 865

HARDING, B, and NELSON, D. H. (1952). Cited in SAMUELS, L. T. and WEST, C D. *Vitam & Horm.*, 10, 288.

[illegible] (1950) *Proc. Soc. exp. Biol. N.Y.*, 73, [illegible]

H[illegible]

H[illegible] 1952.

HELLMAN, L., ROSENFELD, R. S, and GALLAGHER, T. F (1954) *J. clin. Invest.*, 33, 142.

HOOKER, C. W., and FORBES, T. R. (1949). *Endocrinology*, 45, 71.
KAUFMANN, C., and ZANDER, J. (1956). *Klin. Wschr.*, 34, 7.
MCCULLAGH, D. R., and OSBORN, W. O. (1938). *J. biol. Chem.*, 126, 2
MIGEON, C. J. (1956a). *Recent Progr. Hormone Res.*, 12, 398.
MIGEON, C J. (1956b). *J. biol. Chem* 218 ...
MIGEON, C. J. S ...
A
MORRI
PEARL
PEARL
J. biol. Chem, 209, 803. ... PEARLMAN, M. R J., and RAKOFF, A. E. (1954)
PETERSON, R. E., and WYNGAARDEN, J. B. (1956). *J. clin. Invest.*, 35, 552.
PETERSON, R. E, WYNGAARDEN, J. B., GUERRA, S. L., BRODIE, B. B., and BUNIM, J J. (1955). *J. clin. Invest.*, 34, 1779.
RAKOFF, A. E, CANTAROW, A., PASCHKIS, K. E., HANSEN, L. P., and WALKLING, A. A. (1944). *Endocrinology*, 34, 370.
RAKOFF, A. E., PASCHKIS, K. E., and CANTAROW, A. (1943) ... *Obstet. Gynec.*, 46, 856.
SAYERS ...
KE ...
J. c ...
SIRI, W ... (1949). Isotopic Tracers and Nuclear Radiations, Chap 15. New York. McGraw-Hill.
SLAUNWHITE, W R., and SANDBERG, A. A. (1956). *J. clin Endocrin. Metab.*, 16, 971.
SZEGO, C. M., and ROBERTS, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, 61, 161.
WEST, C. D. (1951) *Endocrinology*, 49, 467.
WEST, C. D., TYLER, F. H., and BROWN, H. (1951). *J. clin. Endocrin*, 11, 833.
WEST, C. D., TYLER, F. H., BROWN, H., and SAMUELS, L. T. (1951). *J. clin. Endocrin.*, 11, 897
ZANDER, J. (1954). *Nature, Lond.*, 174, 406.
ZANDER, J. (1955). *Klin. Wschr* 33 ...
ZANDE ...
ZANDI ... *Klin. Wschr.*, 32, 894
ZANDE ... *Klin. Wschr.*, 34, 944.
ZARRO ... , 32, 529 ... and LAZO-WASEM, E. A. (1954). *J. clin. Endocrin. Metab.*, 14, 645.
ZONDEK, B., and BLACK, R. (1947). *J. clin. Endocrin.*, 7, 519.

DISCUSSION

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Pearlman: Yes, I think the point is very well taken. It would modify

the calculations to a certain extent, and what I have presented is perhaps an over-simplification of the problem. It is certainly important to have more information on the metabolism of the steroid hormones by the foetus.

Diczfalusy: I think I have some data which might have a bearing on this problem.

Table I (Diczfalusy)

EXCRETION OF OESTRIOL (μg./l URINE) IN NEWBORN BOYS.
EACH VALUE REPRESENTS A URINE POOL FROM 8 TO 20 BOYS.
(after Diczfalusy, Tillinger and Westman).

Experiment	*Day of life*				
	2	3	4	5	6
1	5700	2700	630	69	121
2	8878	3960	595	48	84
3	5997	3172	386	72	32
4	9300	3287	587	67	
5	4661	1271			
6	8428	3878			
7		1518			
Mean	7162	2827	550	64	79
S.D.	(1942)	(1070)	(111)	(11)	(45)

identified. I might add that approximately 95 per cent of the oestriol was in the conjugated form.

When such newborn boys were injected with oestradiol-17β, very little if any oestrone or oestradiol-17β could be detected, whereas oestriol excretion seemed to be within the normal range (Table II). These data

Table II (Diczfalusy)

EXCRETION OF OESTRONE (OE_1), OESTRADIOL-17β (OE_2) AND OESTRIOL (OE_3) IN NEWBORN BOYS, FOLLOWING THE ADMINISTRATION OF 500 μg OESTRADIOL-17β (IN OIL) ON THE FIRST DAY OF LIFE. EACH VALUE IS EXPRESSED AS μg/l. AND REPRESENTS A URINE POOL FROM 8 TO 20 BOYS

(after Diczfalusy, Tillinger and Westman).

Day of life	*"Free"*			*"Conjugated"*		
	OE_1	OE_2	OE_3	OE_1	OE_2	OE_3
2	0 0	0·0	133·0	1 7	0 0	6110 0
3	0 0	0·0	64 3	0·0	0 0	2200 0
4	0 0	0 0	0 0	0·0	0·0	194·0
5	0 0	0·0	0 0	0·0	0 0	24·0
6	0 0	0 0	0·0	0·0	0·0	18 0
2	0 0	0·0	176 0	2·0	0 0	9706 0
3	0 0	0 0	46·0	0 0	0·0	1777 0
4	0 0	0 0	4 0	0 0	0 0	436 0
5	0·0	0·0	3 9	0 0	0·0	111·0
6	0 0	0 0	0 0	0·0	0·0	31 0

[illegible] in the foetus and [illegible] t. [illegible] ie [illegible] peripheral blood, I have been able to determine these in a [illegible] of domestic animals, and they range from about 0 8 to 0·2 μg. progesterone per 100 ml. plasma. If one takes the available figures for the progesterone content of the human corpus luteum and compares them with the [illegible]

you have a short turnover time this may make a very significant difference to the blood levels.

Pearlman: Yes. The values for *r* by the first two methods indicated would give us the average production rate, and these are not of course the instantaneous production rates, which one should really have.

Bush: I very much agree with Mr. Short I think the fact that you found in practice evidence of two or three 1st order reactions in the

infusions one gets totally different values from the distribution of corticosterone (the endogenous hormone). Furthermore, these turnover times are short compared with the time taken by many substances of smaller molecular weight to reach a steady state in man after infusions.

I think also that your argument that only one measurement is needed on the urinary hormone or metabolite does depend on what class of

time if this is estimated from a single measurement. One would have to take, say, pregnanediol which has a moderate clearance to get reliable figures I think that with progesterone itself you would be likely to be in

by these considerations

Morris The turnover time is really a mathematical rather than a physical picture; I think that is the thing to bear in mind—that it is derived from an overall 1st order reaction, which is just a sum of processes. It is none the less useful.

STUDIES ON THE STEROIDS OF HUMAN PERIPHERAL BLOOD

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ONE of the major difficulties in studying the physiologically active constituents of human blood lies in the small amounts that are accessible through chemical extraction procedures, and the associated problems of acquiring adequate quantities of material for analysis. Some years ago, we attempted to circumvent this problem by directing our attention to the circulating steroids of the bilaterally nephrectomized dog, on the assumption that in the absence of kidneys, or of adequate kidney function, the steroids normally cleared from the blood would accumulate to levels which would permit their ready detection. By studying the dialysates resulting from the treatment of these dogs by the technique of "extra-corporeal dialysis" (Kolff, 1947), we were successful in demonstrating the presence of both free corticosteroid and corticosteroid conjugated to glucuronic acid (Savard, Kolff and Corcoran, 1952). In the intervening years, the presence of both conjugated and non-conjugated corticosteroids in human peripheral blood has been amply demonstrated (Bongiovanni and Eberlein, 1955), and they recently have been elegantly characterized by Vermuelen (1956). The qualitative study of the circulating steroids in situations of anuria or renal failure in the human has, however, remained unexplored. With the generous co-operation of Dr. Warren Guild of the Kidney Laboratory of the Peter Bent Brigham Hospital of Boston a suitable patient was selected for study.

The patient was a 22-year-old woman with acute tubular necrosis resulting from a massive haemorrhage due to an abruptio placenta which occurred at the end of an uncompli-

cated full-term pregnancy. The anuria had been of 8 days duration, and no more than 0 to 50 ml. urine per day had been formed. The patient was not in Dr. Guild's hands until the eighth day and consequently no chemical data relative to circulating steroid levels are available; she was, however, in extreme uraemia with a CO_2-combining power of 12·8 m-moles/l., a blood urea nitrogen of 150 mg. per cent, and a serum potassium of 6–7 milliequivalents/l. The patient was placed on the "artificial kidney" and the peripheral blood subjected to extra-corporeal dialysis (Kolff, 1947) through Visking tubing against a 100 l. bath of rinsing fluid. Two additional dialyses with fresh rinsing fluid served to relieve the patient, who went on to recovery.

The 100 l. fluid from the first dialysis were used in the present study. The method of concentration and extraction of the total steroid fraction was essentially that employed in our first study on dogs and originally described for the isolation of oestrogen conjugates from urine (Grant and Beall, 1950). It consisted in treating the fluid twice with activated charcoal, once with 100 g. (0·1 per cent by weight) and once with 30 g. (0·03 per cent). The combined adsorbates were rinsed first with tap water, then with a little acetone and air dried. The charcoal was suspended in 2 l. pyridine containing 10 per cent water and stirred for several hours. The suspension was filtered and the elution was repeated. The combined pyridine filtrates were evaporated to dryness under reduced pressure and the last traces of solvent removed *in vacuo* over sulphuric acid. The residue was dissolved in 1·5 l. water to give a pale yellow solution, distinctively urine-like in character. Experiments on the recovery of cortisol in varying amounts from 30 l. water (Table I) indicate that at the low concentrations of steroid, a single absorption with 0·1 per cent of charcoal gives 90 per cent recovery (see Levy and Kushinsky, 1954).

The solution (pH 6·5) was extracted five times with 500 ml. ethyl acetate, and exhaustively with methylene dichloride on a continuous extractor. Both extracts were combined and

STUDIES ON THE STEROIDS OF HUMAN PERIPHERAL BLOOD

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ONE of the major difficulties in studying the physiologically active constituents of human blood lies in the small amounts that are accessible through chemical extraction procedures, and the associated problems of acquiring adequate quantities of material for analysis. Some years ago, we attempted to circumvent this problem by directing our attention to the circulating steroids of the bilaterally nephrectomized dog, on the assumption that in the absence of kidneys, or of adequate kidney function, the steroids normally cleared from the blood would accumulate to levels which would permit their ready detection. By studying the dialysates resulting from the treatment of these dogs by the technique of "extra-corporeal dialysis" (Kolff, 1947), we were successful in demonstrating the presence of both free corticosteroid and corticosteroid conjugated to glucuronic acid (Savard, Kolff and Corcoran, 1952). In the intervening years, the presence of both conjugated and non-conjugated corticosteroids in human peripheral blood has been amply demonstrated (Bongiovanni and Eberlein, 1955), and they recently have been elegantly characterized by Vermuelen (1956). The qualitative study of the circulating steroids in situations of anuria or renal failure in the human has, however, remained unexplored. With the generous co-operation of Dr. Warren Guild of the Kidney Laboratory of the Peter Bent Brigham Hospital of Boston a suitable patient was selected for study.

The patient was a 22-year-old woman with acute tubular necrosis resulting from a massive haemorrhage due to an abruptio placenta which occurred at the end of an uncompli-

patient, weight approximately 56 kg., with a presumed normal adrenal output of 25 mg. cortisol per day, it is estimated that

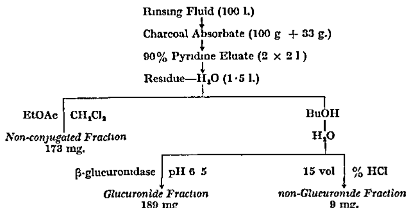


FIG. 1. Steroids from dialysate of human peripheral blood.

at least 200 mg. total steroid should have been released into the peripheral blood since the onset of anuria. It did not seem unreasonable to assume that a proportion of this 200 mg.

Table II

STEROIDS FROM DIALYSATE OF HUMAN PERIPHERAL BLOOD

	Non-conjugated mg	*Conjugated*		*Total mg.*
		β-glucuronide mg	*non-glucuronide mg*	
Corticosteroids (P S [1])	0·65	1·4	—	2·05
17-Ketosteroids (Zimm.[2])	5·5	1·6	0·31	7·40

[1] Porter–Silber reaction (1950)
[2] Zimmermann reaction as modified by Wilson (1954).

should have undergone dialysis and should have been recovered by the extraction procedure. We therefore must conclude that very little steroid indeed had passed from the

Table I

RECOVERY OF CORTISOL FROM WATER
(by charcoal-pyridine procedure—Porter-Silber determination)

Cortisol mg	*Water l.*	*Charcoal (0·1 %) g.*	*90% pyridine ml*	*Recovery mg.*	*%*
100	30	30	3 × 200	58·4	58
10	30	30	3 × 200	7·5	75
5	30	30	3 × 200	4 4	89
1	30	30	3 × 200	1 05	105

washed in the cold with dilute alkali to provide a neutral non-conjugated steroid fraction weighing 173 mg. The aqueous solution was then adjusted to pH 1·0 and extracted several times with *n*-butanol to remove the conjugated steroids according to Cohen and Marrian (1936). On removal of the butanol, the residue was dissolved in 800 ml. water and incubated for 48 hours with bacterial β-glucuronidase (40,000 u.) at pH 6·5 in the presence of chloroform. Continuous extraction at pH 6·5 and again at pH 1·0 with methylene dichloride yielded a neutral conjugated fraction weighing 189 mg. Treatment of the original aqueous solution with 15 volumes per cent of concentrated hydrochloric acid and carbon tetrachloride under reflux according to Rubin and co-workers (1954) provided a non-glucuronide conjugated fraction weighing 9 mg. The extraction and fractionation procedure is shown in Fig. 1.

The steroid content of the free fraction and the two conjugated fractions was determined by the Porter-Silber reaction (1950), and by the Zimmermann reaction as modified by Wilson (1954). The results are given in Table II.

These quantities of steroid, totalling less than 10 mg., were somewhat unexpected. At least they were not expected by the author, for the following reasons. First, in the case of our earlier studies, two dogs yielded a total of 2·9 mg. corticosteroid and 2·2 mg. 17-ketosteroid. In the case of this

(α-ketols) were seen whose absorption spectra in sulphuric acid were unfamiliar. Our attention has been directed primarily to the 17-ketosteroids, whose quantities exceed those of the reducing steroids. These were examined on paper chromatograms in the ligroin-propylene glycol system. The major part of this fraction was found to consist of three individual 17-ketosteroids, whose mobilities indicated the presence of three oxygen functions. Comparisons of chromatographic mobility, alone and in admixture with reference substances, and absorption spectra in sulphuric acid revealed the two major compounds to be 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanolone. In the case of the third, the least in quantity of the three, the results were equivocal between 11-ketoaetiocholanolone and 11-ketoandrosterone. These three compounds were found in *both* the conjugated and non-conjugated fractions. Only one principal steroid was found in the non-glucuronide fraction, and it was tentatively characterized as androsterone on the basis of its chromatographic behaviour and its sulphuric acid chromogen. In all three fractions, several other areas reacting to the Zimmermann reagent were noted, but their small amounts, along with the overwhelming amounts of pigments, discouraged any attempt at characterization (see Migeon and Plager, 1955; and Clayton, Bongiovanni and Papadatos, 1955).

This is the first time that the 11-oxygenated-17-ketosteroids have been observed in human peripheral blood. The accumulation of these compounds is no doubt due to the prolonged anuria and to the fact that the normal catabolites of cortisol and cortisone (tetrahydrocortisol and tetrahydrocortisone and others), which normally would be rapidly cleared from the blood, have not escaped in the present case; these compounds therefore are subjected to a form of biochemical attrition and are further metabolized to the highest point of "steroidal entropy", namely the 17-ketosteroids.

We intend to pursue these studies both as to the number of patients and the directions suggested by these preliminary observations.

patient's blood into the dialysis fluid, in spite of a ready diffusion of the patient's electrolytes.

This raises quite a number of interesting points on which we may speculate. A possible reason for so little steroid in the dialysate is the association between the patient's peripheral steroids (which in this clinical situation are high) and the serum proteins (Eik-Nes *et al.*, 1954); these latter may be so modified by the prolonged anuria that the binding forces are too great to permit dissociation. On the other hand, it may very well be that the anticipated high accumulation of steroid is entirely unwarranted. Perhaps the diffusion space in this patient is much greater than in the normal individual; the data which Dr. Samuels has presented (p. 208) suggest that this is a likely possibility. It may also be that the routes of steroid elimination other than the urine, which are not very important in the normal human, are now compensating for the deficiency in urine formation. Dr. Samuels' group (Migeon, Sandberg and Samuel, 1956) have shown that in the normal human, important quantities of steroid move through the enterohepatic circulation; gastric re-absorption of steroids may thus be entirely suppressed in this patient, and considerable amounts of steroid may be found in the faeces. The sweat, too, may be worthy of consideration in this same regard. These routes will have to be considered in future studies.

We now return to the final aspect of this very superficial piece of work and consider the nature of the individual steroids found in the rinsing fluids from this patient. The conjugated and non-conjugated fractions were examined separately by means of paper chromatography following preliminary purification on silica gel columns. Of the corticosteroids present, cortisone and cortisol have been recognized in the non-conjugated fraction, and tetrahydro E (3α:17α:21-trihydroxypregnane-11:20-dione) and tetrahydro F [illegible]-20-one) in the fraction [illegible] ment with the findings [illegible] r reducing substances

by about one third and then accumulated again on cessation of dialysis

these circumstances.

Savard: Dr. Samuels, have you made any determination of the "diffusible space" in these anuric patients?

Samuels: No, we have not calculated that and we certainly must do so.

Migeon: We have previously reported that the two major 17-ketosteroids in the peripheral blood of normal men were dehydro*epi*androsterone (DHA) (1954. *J. biol. Chem.*, 209, 767) and androsterone (1956. *J. biol. Chem.*, 218, 941) Similar results were obtained by Clayton and Bongiovanni (1955. *J. clin. Endocrin. Metab.*, 15, 693) We also found that the best yields were obtained when using a 48-hour continuous ether extraction at pH 0·8 (1955. *J. clin Endocrin. Metab*, 15, 702).

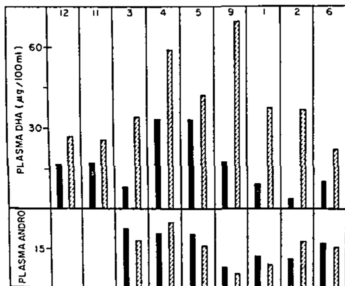


FIG. 1. (Migeon). Levels of dehydro*epi*androsterone and androsterone at the time of delivery in maternal and cord plasma.

The black bars show the maternal values, the shaded bars the cord values. The figures on top of each column indicate the case numbers.

REFERENCES

BONGIOVANNI, A. M., and EBERLEIN, W. R. (1955). *J. clin. Endocrin. Metab.*, **15**, 1524.
CLAYTON, G. W., BONGIOVANNI, A. M., and PAPADATOS, C. (1955). *J. clin. Endocrin. Metab.*, **15**, 693.
COHEN, S. L., and MARRIAN, G. F. (1936). *Biochem. J.*, **30**, 57.
EIK-NES, K , SCHELLMAN, J. A , LUMRY, R., and SAMUELS, L. T. (1954). *J. biol. Chem.*, **206**, 411.
GRANT, G. A., and BEALL, D (1950). *Recent Progr. Hormone Res.*, **5**, 307.
KOLFF, W (1947). New Ways of Treating Uraemia. London. Churchill
LEVY, H., and KUSHINSKY, S. (1954). *Recent Progr. Hormone Res.*, **9**, 357.
MIGEON, C. J., and PLAGER, J. E. (1955). *J. clin. Endocrin. Metab.*, **15**, 702.
MIGEON, C. J., SANDBERG, A. A , and SAMUELS, L. T. (1956). *J. clin Endocrin. Metab.*, **16**, 1291.
PORTER, C. C., and SILBER, R. H (1950). *J. biol. Chem.*, **185**, 201.
RUBIN, B. L., DORFMAN, R. I., and PINCUS, G. (1954). *Recent Progr. Hormone Res.*, **9**, 230.
SAVARD, K , KOLFF, W., and CORCORAN, A C. (1952), *Endocrinology*, **50**, 366.
VERMUELEN, A. (1956). *Acta Endocr* , **22**, 113.
WILSON, H. (1954). *Arch. Biochem.*, **52**, 217.

DISCUSSION

Samuels: In regard to clearance we have made some studies of individuals with renal disturbances and anuria. There is very marked

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group has used—we have a battery of flat areas through which the material flows) and in this 6 hours the high levels of conjugated 17-hydroxycorticosteroids were only lowered by about one-fourth.

In regard to the distribution of compounds, the free steroids show

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levels of conjugated steroids. Furthermore, the change does not occur immediately with diuresis but follows the blood urea nitrogen, as though it were related to the glomerular filtration rate. When one anuric individual was dialysed the conjugated steroids level went down

By using large volumes of blood, of the order of one pint, we have detected 11-ketoaetiocholanolone and 11-hydroxyandrosterone in the glucuronide fraction in very small quantities.

Savard: I am very happy to hear that. This was normal blood?

Kellie: Yes, it was normal. We did about 12 normals, and in three of these we took as much as one pint of blood. We found these two compounds in two out of the three cases.

Migeon: We have had similar experience, Dr. Kellie. We have studied a 500 ml. pool of human plasma. In the free fraction we have detected small amounts of androst-4-ene-3 . 17-dione, DHA and androsterone. In the glucuronoside fraction, we have detected androsterone, aetiocholanolone and 11-ketoaetiocholanolone. However, there was probably not more than 1 μg per 100 ml plasma of each of these 17-ketosteroids.

Savard: In a recent paper by Bongiovanni and Eberlein (1957. *J. clin. Endocrin. Metab.*, 17, 238) the clearance rate of administered androsterone and dehydro*epi*androsterone were compared and a great difference noted. The rates are, I presume, irrespective of their conjugation.

Migeon: Bongiovanni and Eberlein mentioned that following androsterone administration all the androsterone was excreted as a

the androsterone would not be converted to dehydro*epi*androsterone.

Samuels: In Migeon's studies the same conclusions would be logical, that the clearance of dehydro*epi*androsterone conjugate is certainly

cations. The effect is very marked indeed. In a recent paper in *J. Amer. chem. Soc.*, King and Craig have used this as a method of fractionation.

Bush: What pH is this?

Morris: Presumably round about neutrality. The blood would be at neutrality; the pH would be buffered approximately to neutrality.

Savard: A number of people have employed the procedure of enclosing

With this method we have studied the plasma levels of DHA and androsterone at the end of pregnancy comparing the concentration in maternal and cord blood (1955. Johns Hopkins Hosp. Bull, 97, 415). On the upper part of Fig. 1 we have the values for DHA; on the lower part, the values for androsterone. In all cases the values for the cord plasma were higher than those in the corresponding maternal plasma. On the other hand, there was not significant difference for androsterone. It is also to be noted that maternal levels of DHA were lower than the normal average (mean for non-pregnant females : 39·7 µg. per 100 ml. plasma). Gardner and collaborators have also reported a decreased total of 17-ketosteroids in the plasma of pregnant women.

Dr. Savard's patient had been delivered shortly before the blood was undertaken. In view of the results of our own work on 17-ketosteroids in maternal plasma at the end of pregnancy, Dr. Savard has probably studied a rather abnormal situation. This is probably why his data did not mention the presence of DHA in the extracts. Since plasma 17-hydroxycorticosteroids stay elevated for a certain period of time following delivery, there is also the possibility that more corticosteroids might be metabolized to C_{19} steroids; this would partly explain the preponderance of 11-oxygenated-17-ketosteroids observed by Dr. Savard.

Savard: We did not find dehydro*epi*androsterone simply because we did not look for it. We felt that the presence had been competently demonstrated, and furthermore we would have had to use a slightly different hydrolytic condition than the one we employed.

Kellie: With regard to Dr. Savard's flow-sheet dealing with reconstituted urine, I understand that this was a procedure for free steroids, first by manual extraction with ethyl acetate and subsequently by continuous extraction with methylene chloride. Was the possibility covered that the methylene chloride might be extracting conjugates?

Savard: Not in this instance, although had there been any conjugates present they would have been removed with our extract by the subsequent extraction with alkali.

Kellie: I wondered whether this was a possible explanation of the low yield, since our experience is that on continuous extraction with methylene chloride some 17-ketosteroid conjugates are extracted. Did you say that most of the Zimmermann material that was found was these 11-oxy-compounds?

Savard: Perhaps I should have phrased it in another way. They were the most prominent that we encountered, but they certainly did not account for the total amount of 7·4 mg. which was shown by Zimmermann.

Kellie: Does that mean that androsterone and aetiocholanolone were there as minor components?

Savard: I would say, yes; and this is quite unusual.

Kellie: We have made a number of determinations on normal people of the renal clearance of 17-ketosteroid sulphates and glucuronides. The clearance of the sulphates is about 0·5 ml. blood per minute and that of the glucuronides between 200–300 ml. which is extremely fast.

THE PHYSICOCHEMICAL STATE OF CORTISOL IN BLOOD

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Introduction

OUR Chairman's famous slogan, which has been the watchword of this Colloquium, has already received well-deserved approbation; however, I would not like to let this opportunity pass of saying how much "Back to blood!" has been my inspiration in past years. Today, however, I want to deal with an aspect of this sort of work that is only just beginning to receive close attention. With the development of efficient and practical methods for the separation and micro-estimation of steroids, many studies have been made in the last five years on the nature of the steroid hormones in the blood of many species (Lieberman and Teich, 1953; Roberts and Szego, 1955; Hechter and Pincus, 1954). These studies have been mainly if not entirely concerned with the identity and quantity of different steroids present in blood, and with their secretion into venous blood of various endocrine organs. We now know a lot about which steroids are present in blood and in what concentration; we still know very little about how, and at what rates they get out of the blood to their sites of action. It is with an aspect of this last problem that I want to deal today.

Most studies on steroids in blood lead to the conclusion that they are almost entirely confined to the plasma, and that they are easily extracted from plasma with organic solvents. Furthermore, the amounts obtained by extraction with solvents causing considerable denaturation of the plasma proteins are no greater than with those causing little denaturation and relatively little breakdown of lipoproteins, as shown

plasma or serum in a sausage casing, immersing it in a bath and adding steroid to the outer bath, and have found that there is a considerable diffusion through the casing into the protein solution; but of course all these experiments would have been done with free unconjugated steroid, so I think that your point is quite well taken.

Morris: In the original dialysis experiments with this dialysis extraction procedure, there were marked differences of rate (shown by Zaffaroni originally)—differences of transport of different steroids. If the amounts are low I think one always gets some selectivity, a relative rate difference, rather than an absolute barrier difference; but I think it is a fact one must bear in mind before interpreting too clearly.

corticoids although they have an average of 120 days in physiological conditions in which to come to equilibrium with the plasma. The shockingly late realization of the great physiological significance of this fact led to the investigations described in this paper!

Roberts and Szego (1955) have discussed the difficult problem of the terminology to be employed in considering the "binding" of steroids to plasma proteins. Among the requirements they believe necessary before a hormone can be said to be "protein-bound" are relatively constant composition of the lipoprotein complex and the intervention of metabolic processes in its formation. These restrictions seem to serve little purpose since the word "binding" is essentially vague (along with "association", "complexing", "fixation", etc.) unless the free energy of the bonds concerned, or their exact nature, is known. Nor is it certain that all or any types of steroid-protein association of physiological significance will turn out to be catalysed by "metabolic processes" or involve complexes of constant composition. Indeed there is no evidence at present available which is sufficient to prove that any known association between protein and steroid is of constant proportions or involves an enzymic step. Thus, while surviving liver is essential for the formation of "oestro-protein" (Szego and Roberts, 1946), there is as yet no evidence that the "binding" step itself is catalysed by enzymes; it seems more likely that the liver is needed in this system for conversion of the oestrogen to glucuronosides and sulphates.

Since the importance of any "binding" of steroid to protein, or like substances, resides in the effect this is likely to have upon the distribution of the steroid between vascular and extra-vascular compartments, we can avoid terminological confusion by employing a physicochemical parameter, in place of "binding", which is both exactly defined and devoid of any implication as to the particular mechanism involved. This is the chemical activity of the steroid under study. Indeed this parameter must be considered in any discussion of the forces influencing the distribution and transport of

by the low content of total lipid in such extracts. Similarly, the biological activity of whole plasma was accounted for by the extractable steroids present (Bibile, 1953; Bush, 1955). From the analyst's viewpoint, therefore, it appeared that the corticoids and some androgens were in free solution in plasma, and it seemed unlikely that association with other substances in plasma was of great significance.

On the other hand, Szego and Roberts showed that most of the oestrogenic activity of plasma was not ultrafiltrable, and was precipitated with plasma proteins, by salting-out or cold acetone, in a form requiring vigorous hydrolysis before it could be extracted in the cold (Roberts and Szego, 1955) with organic solvents. The significance of this, however, was doubtful since this type of activity was easily dialysable, and besides, the hydrolytic procedures applied to protein precipitates were such as to hydrolyse inactive conjugates. The work of Samuels' group (Eik-Nes *et al.*, 1954; Schellman, Lumry and Samuels, 1954) and of Bischoff (Bischoff and Katherman, 1948; Bischoff and Stauffer, 1954; Bischoff, Stauffer and Gray, 1954) however, showed clearly that the solubility of various steroids in protein solutions was greatly increased. They showed clearly that considerable amounts of steroid were "bound" to albumin although the association was easily broken by organic solvent extraction. This work, however, seemed to indicate that the less polar steroids and the phenolic steroids were associated more firmly and in greater quantity than were the corticoids.

Evidence that the chemical activity of cortisol and corticoids in plasma might be much lower than in free aqueous solution was provided by the early dialysis studies of Zaffaroni (1954). Again the physiological significance of this was in doubt since room temperature made the achievement of equilibrium very slow and might give results very different from those pertaining *in vivo*. One very simple consideration, however, provided a sound reason for believing that the chemical activity of cortisol in plasma was extremely low; this was the fact that fresh red blood cells contain little or no detectable

solvent with a small pipette when necessary. When an even line not wider than 1·5 mm. had been obtained the strip was dried quickly with a hot air blast. It was stored in a small warm dry container until counted; this avoids thickening of

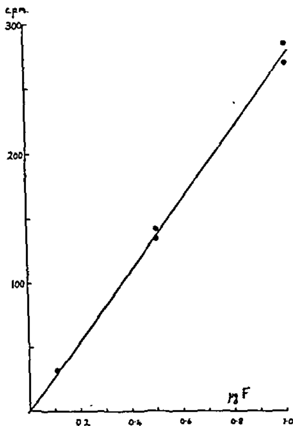


FIG. I. Strip counting of [4-^{14}C]cortisol.

the area occupied by the extract due to absorption of moisture. Although this method gives counts at infinite thickness it is ideal when large numbers of extracts have to be counted. The strip was mounted vertically in a frame fitting the counter's sample chamber so that the top edge was 2–3 mm. below the

the steroid hormones, whatever other terms and concepts are introduced. In practice the measurement of the activity of non-electrolytes in very dilute solution is difficult; the exact relevance of the term in the following experiments will be reserved for the discussion.

Methods and Results

The general procedures were as described before (Bush, 1952, 1953, 1954) and only new techniques or modifications will be described here.

Freely extractable cortisol was measured by extraction with ether: ethyl acetate (2 : 1 v/v) instead of ethyl acetate. Chromatograms of extracts containing much lipid were prepared by a new technique; instead of running-up the extract to the origin of the chromatogram (Bush, 1952) with ethyl acetate: methanol (3 : 1 v/v) or similar solvent, it was run up twice with 80 per cent aqueous methanol to 2 cm. above the top of the area over which the extract was deposited. This carries up the steroids but leaves behind the mass of non-polar lipids. The area containing the latter is cut off and the steroids then run up to the origin of the chromatogram with ethyl acetate: methanol as usual. This technique is effectively a preliminary reversed-phase chromatogram (Morris and Williams, 1955), in which the non-polar plasma lipids themselves form the stationary phase.

Radioactive cortisol was estimated by an extension of the running-up technique. The extract or eluate of a chromatogram was deposited on a strip of Whatman No. 2 paper 15 × 1·25 cm. using ethyl acetate: methanol. Five µg. (non-radioactive) cortisol was then deposited as carrier and a small amount of Waxoline purple (I.C.I. Ltd.) as fat-soluble dye. It was then run up with ethyl acetate: methanol or 80 per cent methanol to the top of the strip and some evaporation allowed so that concentration of the dye and extract occurred for 10–15 minutes at the extreme top edge of the strip. Unevenness of the line of dye was corrected by manual deposition of

ments but showed little variation within any one experiment; this is largely due to the fact that our technique routinely involves rapid batch-extraction of ten samples at a time in a special apparatus, and very rapid evaporation of extracts eight at a time, each batch taking 8–15 minutes for complete evaporation. All recovery controls were carried out with quantities of [4-^{14}C]cortisol similar to and/or less than those used in each experimental sample (0·5–2·0 μg.). Because of small losses when this quantity of cortisol was incubated in aqueous solution in glass vessels, control samples were incubated under the same conditions for the same length of time as the test samples. Typical calibration curves are shown for pure [4-^{14}C]cortisol in Fig. 1, and for pure and extracted cortisol in Fig. 2.

Pilot experiments

These were carried out with non-radioactive cortisol, or plasma without any added steroid. Part of a fresh heparinized sample of venous blood from a healthy donor or from patients without endocrine disease was centrifuged within 15 minutes of collection (and the plasma and cells collected separately). The cortisol was estimated in the cells, plasma, and whole blood, and a haematocrit taken. No cortisol was detected in the cells of 14 out of 15 samples of blood, and only a trace in the fifteenth. On the other hand, the plasma concentration obtained by using the haematocrit and the measured blood concentration was invariably lower than that measured directly by extraction of the plasma, sometimes by as much as 50 per cent of the latter value. The explanation of this resulted from later experiments.

Dialysis of heparinized plasma (Visking 36/32″) against 3–4 volumes of Krebs solution at 37° for 8 hours was next carried out. Apparent distribution coefficients ranged from 6 0 to $>12 \cdot 0$. Incubation of plasma with cortisol or cortisone for 8 hours at 37° led to no interconversion of the two compounds, nor apparently any conversion to other steroids in detectable amounts.

centre of an end-window counter having a 44 mm. diameter window. Slight differences in self-absorption were noted with extracts of greatly different purity (for example, extracts of salt solutions compared with those of plasma), so that all

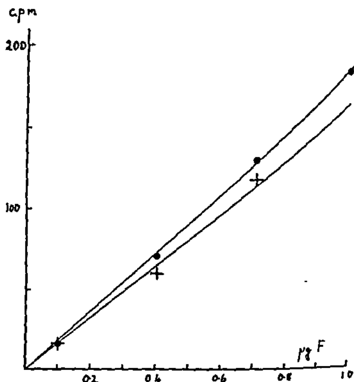


FIG 2. Calibration for [4-^{14}C]cortisol (F).
●—counted directly.
+—counted after standard extraction and chromatography.
Mean recovery 89 per cent.

experiments included control extracts of [4-^{14}C]cortisol added to the appropriate medium; the results for each type of medium were then calibrated by the recovery experiment for that medium. All results given below have therefore already been corrected for both extraction and counting error. The recoveries varied from 70–100 per cent in different experi-

large fall in C_1/C_0 at 24–25 hours. It was noted that in each experiment with plasma the outer solutions were clear at 8 hours but cloudy at 24–25 hours. It was also noted that a second experiment with plasma RB II (Table I) one week

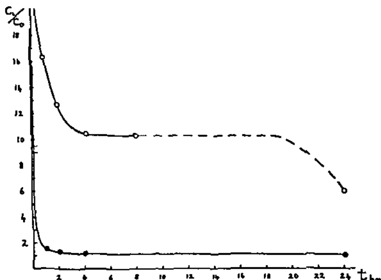


FIG 3 Dialysis of cortisol.
Sac volume : 20 ml.
Outer volume : 30 ml
Total quantity of steroid : 3 μg.
Temperature : 37–38°.
○—plasma in sac } 0·9 % NaCl
●—0 9 % NaCl in sac } outside

later gave a C_1/C_0 value of 1·56 at 25·2 hours compared with a value of 8·72 when fresh.

It was concluded from these experiments that there was present in normal human plasma a component which reduced the chemical activity of cortisol to about one-tenth its value in dilute saline solution. This component was relatively unstable, showing evidence of disappearance of its effect on cortisol within 24 hours at 37–38° *in vitro* and within one week at 2–4°. At each temperature this loss of effect was correlated

Experiments with [4-^{14}C]cortisol

Dialysis: 0 5–2·0 μg. [4-^{14}C]cortisol were added to 10–20 ml. citrated plasma and allowed to equilibrate by shaking at 37–38° for 1–2 hours. The plasma was then placed in a sausage of Visking 18/32″ tubing closed with six knots and dialysed against 20–30 ml. 0·9 per cent NaCl. Experiments were carried out to determine the time course of the passage of cortisol into the outer solution, and the concentration ratio (inside/outside, C_i/C_o) at equilibrium. Table I shows some of

Table I

Plasma Sample	*C_i/C_o at 25 2 hours*
W	7·95
L	5·10
B	11·70
R	8 78
RB II (*a*)	8·72
RB II (*b*)	1·56

3 μg cortisol in system.
Sac volume: 20 ml. (Plasma; 18/32″ Visking Tubing).
Outer volume: 30 ml (0 9 per cent NaCl).
Temperature. 37–38°.

the values obtained with different plasmas after 25·2 hours at 37–38°. The results showed a considerable reduction in the chemical activity of cortisol relative to that in saline, but were somewhat variable. This seemed to be correlated with a variable degree of cloudiness in the outer solution, and an increase in the amount of total lipid in the extracts of both compartments over that found with fresh plasma extracts. It seemed reasonable to infer that considerable breakdown of lipoproteins was occurring during incubation.

Progress curves, of which Fig. 3 is typical, showed that equilibrium was achieved rapidly between saline and saline. On the other hand an apparent equilibrium achieved between plasma and saline at 4–6 hours (37–38°) was followed by a

that at least two components or chemical groups which reduce the chemical activity of cortisol are present in plasma. One of these has a high affinity for the steroid but is saturated at low concentration, the other(s) have a lower affinity but have a very large capacity since the isotherm is linear up to an equilibrium concentration in plasma (C_1) of at least 120 µg./100 ml. Since none of these components is dialysable through cellophane it is likely that distribution of cortisol across undamaged capillary walls is governed by an isotherm of similar form. In this connection it is interesting that the component of high affinity becomes saturated at just about the upper limit of normal plasma concentration of cortisol in man. Since plasma concentrations of this steroid in man rarely rise above 60 µg./100 ml., even after infusions of ACTH, it seems unlikely that the concentration of cortisol in extracellular fluid ever exceeds 10–15 µg.

The nature of these components of normal human plasma was further investigated by experiments on purified plasma protein fractions prepared by a method similar to Cohn's, but using ether as the organic precipitant. This method is the one employed by the Lister Institute, and the fractions were kindly supplied through the courtesy of the Director, Dr. A. A. Miles. Exhaustive extraction of three of these fractions with organic solvents first at 45°, then at room temperature, was carried out and the extracts examined by paper chromatography for their content of cortisol. The results are shown in Table II. About half the amount of cortisol, expected to be

TABLE II

CORTISOL CONTENT OF PURIFIED PLASMA PROTEIN FRACTIONS

Fraction	*Weight* g.	*Equivalent Vol. Plasma*	*Cortisol* µg.
(G 3) γ-Globulins	0 4	40	0 0 (< 0 13)
(F.I W.) Fibrinogen	0 2	40	0 05
(G 2) α- and β-Globulins	0 8	40	1·0

with a great increase in the total lipid extracted by the standard technique which was attributed to breakdown of lipoproteins, since dialysable lipid was released.

Since all the previous experiments had been carried out with total steroid concentrations in the inner compartment well within the physiological range of plasma concentration, it was necessary to determine distribution isotherms to gain further information about the plasma component having this

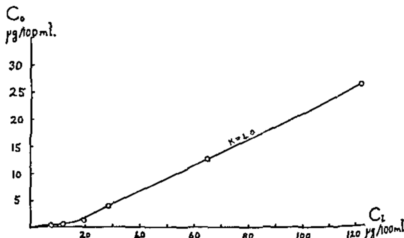


Fig. 4. Plasma/saline distribution isotherm (C_i/C_o).
Temperature : 37–38°
Time : 9 25 hours.

striking effect upon the chemical activity of cortisol. Four of these have been constructed to date, all of them remarkably similar. Fig. 4 shows one of these, which has been corrected for the dilution effect of citrate, by assuming that the small concentration change involved produces no significant change in the activity coefficients of the components under study. It is seen that up to an equilibrium concentration in plasma (C_i) of 20 μg./100 ml. C_i/C_o is similar to that found in the earlier experiments, namely approximately 10. Above this value, however, the isotherm becomes linear with a distribution coefficient in favour of plasma of about 4·0. This suggests

the total lipid of the extract was large compared with that from an equivalent volume of fresh plasma. These negative results had been anticipated in view of the known instability of the plasma components concerned in the reduction of cortisol activity.

Distribution between Red Cells and Plasma

The pilot experiments of this series had confirmed previous experiments undertaken from the analytical point of view by many workers. However, it was felt desirable to attempt to determine the exact distribution ratio of cortisol between plasma and red cells by using the radioactive steroid to attain maximum sensitivity for detecting the presumably minimal amounts in the cells. In order to minimize the effects of metabolic processes upon the distribution, the first experiments were carried out by using saline, or citrated plasma, containing fluoride to inhibit glycolysis. The red cells were washed three times in saline-citrate before resuspension in fresh saline or citrated plasma which had been equilibrated for an hour with [4-^{14}C]cortisol (2 μg.). The suspensions were then incubated at 37–38°. Later the same experiments were repeated, this time omitting fluoride and using glucose-saline buffer to ensure the maintenance of normal ionic composition of the cells.

To the author's surprise the total recovery of cortisol from the system was extremely low, although control recoveries from saline and plasma were in the normal range (70–100 per cent in different experiments). In view of the latter, C_i was calculated by difference from C_o since the measurement of the latter seemed reliable. It seemed that cortisol was rapidly taken up by ("on" or "in" is as yet undecided) the cells, although equilibrium was not surely obtained in the fluoride-treated samples at 14 hours when haemolysis made further incubation unreliable. In contrast, the samples without fluoride, and in the presence of adequate glucose, achieved equilibrium in 2 hours (Table IV) with a far greater proportion of the steroid taken up by the cells. The difference

found in the volume of plasma containing the weight of plasma proteins extracted, was accounted for. Nearly all of this was in the fraction G.2 (α- and β-globulins, comprising all the known lipoproteins). These results were not considered of more than superficial interest, however, since co-precipitation is by no means an indication of association in solution. Solutions containing concentrations of the different protein fractions close to those found in normal plasma were therefore prepared in 0·9 per cent NaCl, and [4-^{14}C]cortisol equilibrated with the solution for an hour prior to dialysis against saline at 37–38° for varying times. The results of a typical experiment are shown in Table III. No reduction of the activity of

Table III

Distribution of Cortisol between Protein Solutions and 0 9 per cent Saline

	C_i/C_o		*Protein conc. % wt./vol.*
	6 25 hr.	*25 5 hr.*	
(F.I.W.) Fibrinogen	2 81	2·29	0·5
(G.2) α- and β-globulins	1 44	1 56	1·06
(G 3) γ-globulins	1 42	1·19	0·66

Sac volume: 10 ml (Visking 18/32" tubing).
Outer volume: 20 ml.
Added steroid 2 0 μg.
Temperature: 37–38°

cortisol comparable to that seen in plasma was found with any fraction, although a significant reduction was found in all three. This was considerable in the case of fibrinogen, but very small with the other two. The results with the fraction G.2 are of little use from the physiological point of view since this fraction is partially denatured by the process of preparation and is only partly soluble on reconstitution. This fraction behaved like plasma at 24 hours at 37°, namely a large amount of lipid was freely dialysable, even at 4–6 hours, and

in favour of the lysed cell solution, and also that the recovery of cortisol from this solution was very low. When this was allowed for, the measured values of C_i agreed with those calculated from the measured values of C_o.

Discussion

The main purpose of the above experiments was to determine the influence of non-dialysable components of plasma upon the chemical activity of cortisol in plasma, and thus to get some idea of the probable distribution of this hormone between the blood and tissue fluids. For the reasons given in the introduction it was decided to concentrate upon a simple method of measuring the chemical activity of cortisol in plasma or a simple function thereof. The value of the dialysis method seems to be that the function measured is likely to be more closely related to the actual distribution of cortisol *in vivo* than any other that can be measured. Before discussing the physiological significance of the results described above it is necessary to enquire into the exact nature of the parameter measured by dialysis-equilibrium experiments. In dealing with this point it should be remembered that the chemical activity of cortisol, in the media used in these experiments, influences both the distribution coefficient between the two phases separated by the dialysis membrane and the quantity extracted from these phases by the organic solvent used in the analytical method for estimating this compound If, therefore, cortisol formed a reversible association with a dialysable substance, such that its activity was reduced to a small fraction of that in saline, less "freely-extractable" cortisol should be obtained on extracting each phase of the system. If, however, the extracting solvent itself formed an association with the substance associating with cortisol, or caused an alteration in its structure that changed the equilibrium constant of its association with cortisol, this would not occur. Further complications might arise depending upon whether the complex formed were extractable, and whether or not the

Table IV

DISTRIBUTION OF CORTISOL BETWEEN ERYTHROCYTES AND SUSPENDING MEDIUM

	C_i/C_o		
	2 hours	4 hours	14 hours
0 9 per cent NaCl[1]	0 84	1 8	2 0
Saline-glucose buffer[2, 3]	5·23	—	—
Citrated plasma[1]	0 30	0·44	0·50
Citrated plasma[2]	1 76	—	—

(1) Fluoride added.
(2) pH 7 5.
(3) O_2 : CO_2–95 per cent : 5 per cent bubbled through

between the distributions with saline and plasma was in each case in the direction expected in view of the known reduction of activity in plasma.

The significance of these results was doubtful in view of the low total recovery of cortisol. Packed cells were therefore washed, repacked, diluted with water to give a solution containing 6·0 g./100 ml. haemoglobin and centrifuged to remove "ghosts". [4-^{14}C]Cortisol (2·0 μg.) was added to 10 ml. of the solution and, after equilibration at 37–38° for one hour, dialysed against 20 ml. saline or plasma. Controls were set up of the solution of lysed cells, saline, and plasma. The results are given in Table V. It is seen that distributions were

Table V

DISTRIBUTION OF CORTISOL BETWEEN LYSED RED CELL SOLUTION AND PLASMA OR SALINE. RECOVERIES FROM LYSED CELLS

Plasma outside: C_i/C_o = 1 06.
Saline outside: C_i/C_o = 2 55.
10 ml. lysed cell solution (Hb concn., 6 0 g./100 ml.).
20 ml. outside solution.
Visking 18/32" tubing.
37–38° 8-hour incubations.

Recovery of cortisol from lysed cell solution:
Stored at 0° for 8 hours: 35 per cent.
Incubated 37–38° for 8 hours: 23 per cent.

normal plasma is albumin (as electrophoretically defined). This conclusion, however, needs checking since his experiments were done with heparinized plasma and in the cold. The former affects the mobility of certain lipoproteins; the latter could affect the dissociation constants of steroid protein complexes.

If the distribution of cortisol between blood and extra-cellular fluid normally approximates to an equilibrium, or involves a steady state in which the passage of steroid across the capillary membrane is a rate-limiting step, then the above results suggest that, despite their easy extractability from plasma with organic solvents, the corticoids will reach the tissues at concentrations and rates far below those expected from their plasma concentrations. As in the case of thyroxine, the magnitude of this effect is such that one can hardly doubt that the tissues' concentration of cortisol in man depends as much upon the concentration and properties of the plasma component with which it associates as upon the concentration of the steroid itself. It seems reasonable at present to accept this hypothesis since there is evidence to hand that the concentration of cortisol in extra-cellular fluid is much lower than in plasma (Cope and Hurlock, 1953; Baron and Abelson, 1954; Sandberg *et al.*, 1954) but rises towards plasma levels in the presence of local inflammation. Baron and Abelson (1954) found approximately 0·2–0·4 µg./100 ml. cortisol in normal cerebrospinal fluid: if the distribution between plasma and C.S.F. were entirely determined by the isotherm of Fig. 4 this would be expected with a plasma concentration of 5–10 µg./100 ml. cortisol, namely the normal range. Although the blood-C.S.F. boundary is more selective than that for tissue fluids in general, the close correspondence of these two sets of results suggests very strongly that the distribution of cortisol between tissues and plasma follows closely that found in the model systems *in vitro*.

While the present experiments seem to have thrown some light on the distribution of cortisol between plasma and extra-cellular fluid, they have not yet progressed far enough to

solvents of the chromatographic system influenced its dissociation constant. In the case of plasma and the plasma protein fractions there was no evidence of any such phenomenon. This, however, only enables us to fix an upper limit to the chemical activity of cortisol in the phases examined here, and does not enable us to determine the exact function relating chemical activity to the concentrations of "freely-extractable" cortisol measured.

Here in fact lies the real advantage of the dialysis equilibrium method; while it does not measure the exact chemical activity (in terms of that in a standard phase) of the steroid under investigation, it determines the activity of the steroid and all its dialysable complexes. It thus should give the best approximation available of the activity of all those forms of the steroid which are likely to be freely available to extravascular tissues. While any such complexes are obviously, from the above results, either lipid-soluble or easily split by [illegible] f them are [illegible] steroid in [illegible] e complex of cortisol that exists in normal plasma is dissociated by cold extraction with ether-ethyl acetate, but it is stable in plasma for at least 8 hours *in vitro* at 37–38°.

With these limitations of the method in mind it seems reasonable to refer to "dialysable" and "non-dialysable" cortisol in plasma, and to conclude that the former represents an upper limit to the quantity of hormone capable of leaving the blood and reaching the tissues. Daughaday (1955, 1956, 1957*a*, *b*) has obtained similar results, the full account of which only appeared during the writing of this paper. However, his results were obtained at 4° and in some respects may not tally with the present work because of this. He also concludes that cortisol has such a low chemical activity in plasma (expressed as "binding") that it may not be as freely available to the tissues as formerly believed. He has inferred, from the results of an extremely elegant method of equilibrium electrophoresis, that the component responsible for this in

results above suggest that an unstable lipoprotein of the α- or β-globulins is the responsible component, but this conclusion is not justified at present.

Acknowledgements

grateful. He is also indebted to numerous colleagues, especially Dr. Rosemary Biggs and Dr W. Cranston, for supplies of human blood and plasma.

REFERENCES

BARON, D. N, and ABELSON, D (1954) *Nature, Lond*, **173**, 174.
BIBILE, S. W (1953). *J. Endocrin*, **9**, 357
BISCHOFF, F., and KATHERMAN, R. E. (1948) *Amer J. Physiol.*, **152**, 189.
BISCHOFF, F, and STAUFFER, R D (1954). *J Amer chem Soc*, **76**, 1962.
BISCHOFF, F., STAUFFER, R. D., and GRAY, C L. (1954). *Amer. J. Physiol.*, **177**, 65.
BUSH, I E (1952). *Biochem, J.*, **50**, 370.
BUSH, I. E (1953). *J. Endocrin*, **9**, 95.

DAUGHADAY, W. H (1957*a*) *J. clin. Invest*, **35**, 1428
DAUGHADAY, W. H. (1957*b*) *J clin Invest*, **35**, 1434
EIK-NES, J., SCHELLMAN, J A, LUMRY, R, and SAMUELS, L. T. (1954). *J. biol Chem*, **206**, 411
HECHTER, O., and PINCUS, G. (1954). *Physiol. Rev*, **34**, 459.
LIEBERMAN, S, and TEICH, S. (1953). *Pharmacol Rev.*, **5**, 285.
MORRIS, C J. O R., and WILLIAMS, D C (1955). Ciba Foundation Colloquia on Endocrinology, **8**, 157
ROBERTS, S., and SZEGO, C. M (1955). *Annu Rev Biochem.*, **24**, 543.
SANDBERG, A A., EIK-NES, K, NELSON, D. H., and TYLER, F. H. (1954). *J Lab. clin Med*, **43**, 874
SCHELLMAN, J A., LUMRY, R., and SAMUELS, L T. (1954). *J. Amer. chem Soc*, **76**, 2808
SZEGO, C. M, and ROBERTS, S. (1946) *Proc. Soc. exp. Biol, N.Y.*, **61**, 161.
ZAFFARONI, A. (1953). *Recent Progr. Hormone Res.*, **8**, 51.

demonstrate the factors determining the entry of this steroid into cells themselves. The distributions shown in Table IV cannot be explained by the presence of occluded plasma, nor by changes in volume of the cells. One possible explanation of the contrast between these results and those of previous experiments, by the author and others, is that washing removed the surface albumin of the cells which was partly replaced on resuspending in plasma but not, of course, in saline. However, these experiments are mentioned in order to caution those who wish to estimate cortisol in red cells; by our method recovery of cortisol from lysed cells, even when diluted 1 in 5, was only 30 per cent at concentrations of 5–20 μg./100 ml. While this may be a feature peculiar to our extraction method it suggests that it would be worthwhile repeating previous experiments on red cells in order to check that the failure to find cortisol in them was not due, as in the author's case, to very low recoveries. In any case the significance of C_i/C_o is in doubt in these experiments since C_i was calculated from C_o and does not in this case represent "freely extractable" cortisol.

The experiments reported here, and those of Daughaday (1957*a*, *b*) provide a simple physicochemical explanation for the low concentrations of cortisol in human extracellular fluid and suggest that the phenomenon will prove to be a general one, and not simply a consequence of the rather special examples of this fluid which have been examined to date. Both sets of experiments have shown that two non-dialysable components (at least) are responsible for the low activity of cortisol in plasma. It seems likely to be of some significance that the component forming a complex of low dissociation constant appears to become saturated just at the upper level of the normal range for plasma cortisol concentrations. The nature of this component is believed to be in doubt at the moment since, although Daughaday's electrophoresis experiments suggested it was albumin, purified albumin did not associate strongly enough with cortisol to account for the effect found with plasma (Daughaday, 1957*a*, *b*). Some of the

top of the packed RBC. In order to determine exactly how much plasma was left, a microhaematocrit (using Aloe 23920 Hematocrit Centrifuge and centrifuging at 10,000 rpm for 5 minutes) was run on each RBC sample. There is still about 5 per cent with the red cells when one runs an haematocrit with the Wintrobe tube, and approximately 1 per cent with the microhaematocrit

recovery.

In our *in vitro* experiments, neither time nor temperature seemed to influence the results.

It is not possible to tell at the present time if the steroids are adsorbed on the red cell membrane or if they actually penetrate into the cell.

experiments of a similar nature on the uptake of a variety of other drugs by the red cells.

Sonenberg. Dr. Bush, you showed us a distribution isotherm for plasma/saline. I wonder if you have a similar isotherm for saline/saline You just showed us one concentration for saline.

Bush· That was simply a control on the achievement of equilibrium between saline and saline.

Sonenberg· I had in mind the work, referred to by Prof. Morris this

DISCUSSION

Migeon: We too have been working on the distribution of cortisol between plasma and red blood cells (RBC)

We have carried out several sets of experiments; one of them was an *in vivo* study (Migeon, Lawrence, Wall, Bertrand and Holman, in preparation). Cortisol (1 mg./kg. body weight) was administered intravenously over a 30-minute period and blood samples were collected 1, 2, 4 and 6 hours after the beginning of the infusion. Each sample was centrifuged for 30 minutes at 2000 rpm. and the plasma was removed as completely as possible. By running a microhaematocrit on the RBC fraction it was observed that this fraction contained 5–10 per cent of its volume of plasma. After applying a correction for the plasma left with the RBC's, we found that 30–40 per cent of the total free 17-hydroxycorticosteroids in a given sample of whole blood was with the RBC fraction.

We also studied the effect of normal saline washings (4 times with once the volume of RBC) on the amount of free 17-hydroxycortico-

explains why in our previous work with [4-^{14}C]cortisol (1956. *J. clin. Endocrin. Metab.*, **16**, 1137) there was very little activity in the RBC fraction, while Peterson and associates (1955. *J. clin. Invest.*, **34**, 1779) reported that 20–25 per cent of the activity was in the RBC and 75–80 per cent in plasma. Since we had washed the RBC twice with twice their volume of saline, we had been removing most of the activity accompanying the red cells.

In our *in vivo* work, we also studied the 17-hydroxycorticosteroids liberated by β-glucuronidase hydrolysis. After proper correction, no

What were the amounts in the cells compared to plasma when you centrifuged the non-radioactive material immediately and analysed and identified the spots?

Bush: With non-radioactive material centrifuged within ten minutes of collection, we were able to detect cortisol in the lysed cells only in one sample, but when we subsequently tested the recovery of cortisol from lysed cells, rather than from plasma, the recovery was extremely low. One-fifth dilution of lysed cells reduced our recovery to 30 per cent. I do not know whether this happens in your method. Have you ever done recovery of radioactive or free cortisol added to lysed cells, [illegible]

ot your glucose buffer system?

Bush: We have not done that, but we have followed very carefully the conditions established by Maizel and Harris for this sort of experiment, and our experience is that in conditions where you use fluoride the rate of entry of cortisol into these cells is far slower and achieves a far slower equilibrium concentration inside than outside. Then when these cells are incubated in the absence of fluoride, in the presence of added glucose and in the presence of a fully buffered Krebs saline solution, all the evidence of many people goes to show that electrolyte exchange is perfectly normal, or at any rate relatively normal; certainly very much better than in the fluorided cells When you use fluorided cells you get leakage of electrolytes. So although we have not yet checked the concentration of sodium inside I should be very surprised, judging by the results of Harris and Maizel, if the running down of the cells is worse in the second solution.

Prunty. It should be done.

Bush: I agree.

effect would not be seen even if the molecules were charged ones. The maximum concentration which we used was 125 μg./100 ml., that is about 4 μM, at which I do not think this sort of effect is seen.

Morris. I think it might not be appreciable, but I think that on the other hand what Dr. Sonenberg suggests would be worth doing.

Bush: I agree.

Tait: Regarding the penetration of [4-^{14}C]cortisol into blood cells, we continually collect blood at room temperature with [4-^{14}C]cortisol in the bottle. It remains for 30 minutes before centrifuging and is probably well shaken in the journey to the centrifuge. The recoveries,

missing radioactivity has entered the cells; this would support Dr Samuels' concept of active non-transport.

Bush: I doubt very much wheth
with 30 minutes between the beds
blood quite under our conditions
movement) at about 2 cycles per second for one hour at 37°, so that you do get completely adequate mixing.

But if you have not got adequate mixing of your added control radioactive cortisol, you cannot use that as a comparison for what is happening with the cortisol which is present at the start. My experience has been that if you add small volumes of radioactive cortisol, or even non-radioactive cortisol, in saline solution to plasma, and you let your technician carry it up and down the room once or twice and then leave it on the bench for 30 minutes to equilibrate it, then you do get very high recoveries very readily. It was only when we started doing experiments in which we really shook the solution up to get really good mixing that we discovered this very low recovery.

Tait: In
for collect
the same.
Dr. Samuels has said.

Bush. Yes, except that at 0° it is well known that these relatively small changes in red cell volume cause enormous changes in permeability of red cells. One only needs to get 1·7 times the original

individual.

protein, in the course of transport, or at postulated reactive sites in the target organ, may be an important key to the mechanism of action of the steroid hormones.

Oestroprotein Formation *in vitro*

The oestrogenic lipoprotein complexes described in the blood of several species (Szego and Roberts, 1946) may have been reproduced in an *in vitro* model system which has been described from these laboratories (Szego, 1953). An enzymic mechanism capable of promoting the association of ^{14}C-labelled oestrone and oestradiol or their isotopic metabolites with homologous serum protein was demonstrated in surviving rat hepatic tissue. The activity was confined to liver, among several tissues studied, and was correlated with the functional status of the preparation, being high in liver regenerating after partial hepatectomy (Szego, 1953), and minimal in primary and transplanted hepatoma (Szego, 1955). These studies were initially carried out by cold acetone precipitation and fractionation of the serum incubation medium along the lines summarized in Fig. 1, and analysis of the fractions for radioactivity and oestrogenicity. In later investigations (Szego and Roberts, 1955*a*, 1956), a paper electrophoretic method of resolution of the incubation medium was developed, by means of which distribution of radioactivity by scanning in an automatic windowless gas-flow counter, and of protein by staining subsequently with bromophenol blue, could be determined on the same paper strip. Correlation of these data permitted identification of the serum proteins which participated in the binding process. In the absence of surviving liver tissue, radioactivity was confined to an area behind the most slowly migrating proteins and thus, unbound. When incubation had been performed in the presence of surviving liver tissue prepared by free-hand mincing, association of radioactivity derived from [16-^{14}C]oestrone or [16-^{14}C]oestradiol occurred selectively with the albumin area, with unbound (residual) radioactivity migrating as above. The use of splenic tissue yielded data which were indistinguishable from the

STEROID INTERACTION IN THE *IN VITRO* BIOSYNTHESIS OF STEROID-PROTEIN COMPLEXES*

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STUDY of circulating forms of the steroid hormones, in context with physiological observations, may be expected to yield in due time valuable clues to the mechanisms which underlie steroid action *in vivo*. The information thus far available has been reviewed recently from these laboratories (Szego and Roberts, 1953; Roberts and Szego, 1953, 1955; Szego, 1956), wherein evidence was summarized for the critical function of the liver in two apparently related phenomena:

1. The *in vivo* activity of certain steroid hormones and their interactions in inducing modification of target organ structure and function.
2. The biosynthesis of protein-bound transport forms of steroid hormones.

It is not the purpose of this discussion to summarize the evidence linking these phenomena. Rather, it is intended, first, to present extension of previous observations on oestro-protein formation *in vitro* to the investigation of hepatic [illegible] in the association of cortisol and corticosterone or [illegible] ide evidence [illegible] strogens and [illegible] tein-binding phenomenon; and finally, to attempt to examine the compatibility of these data with the hypothesis that association with

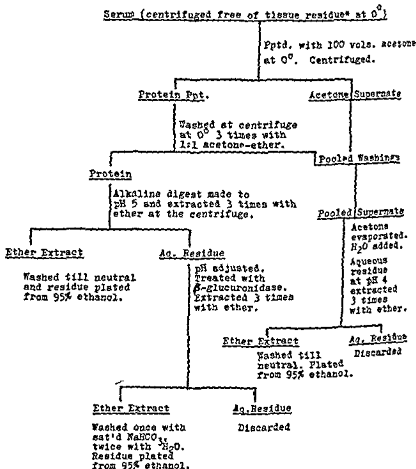
* Aided by a grant from the National Cancer Institute of the National Institutes of Health (C-1488), and by Cancer Research Funds of the University of California.

serum control. Thus, in the case of the oestrogens, the presence of liver tissue in the incubation medium was obligatory for protein binding *in vitro*.

Studies on the nature of the bond have thus far provided meagre data. Like the endogenous circulating oestrogenic lipoprotein (Roberts and Szego, 1946), oestroprotein formed *in vitro* appears to occur as a glucuronosidic complex associated with protein carrier. This assertion is made on the basis that hydrolysis either with hydrochloric acid or with β-glucuronidase serves to release ether-soluble oestrogenic radioactivity from the partially digested serum protein in the case of the material formed *in vitro* (Szego and Wolcott, 1955).

The specific localization of isotopic oestrogen or its metabolites with albumin under these conditions has been substantiated by cold ethanol fractionation of the serum incubation medium and demonstration of radioactivity exclusively in the albumin fraction (Szego and Roberts, 1956).

It is further borne out by an experiment depicted in Fig. 2, in which the mince preparation was incubated in the presence of [16-^{14}C]oestrone, in a Krebs Ringer phosphate buffer instead of in whole serum. A purified rat serum albumin preparation, containing a trace of β-globulin as a contaminant, was added to the medium in the amount of 2 per cent to serve as potential protein carrier. Here again albumin appeared to transport the bulk of that radioactivity which was associated with the protein moiety. It should be stressed that albumin appears to occupy this rôle under conditions which require *bulk* transport of several hormones of varying chemical constitution (and of many other quite unrelated materials), both *in vivo* and *in vitro*, especially when the capacity of specific carriers has been exhausted (cf. Roberts and Szego, 1955; Robbins, Petermann and Rall, 1955). It should be noted further that albumin has been shown to serve in a similar capacity in accepting fatty acids during the enzymically-catalysed heparin clearing reaction *in vitro* (cf., Anfinsen, 1954; Robinson and French, 1953). Nevertheless, albumin, exclusively, may not serve this function in the case



*Liver tissue fractionated along similar lines, with exception that aqueous residue from ether extraction of alkaline digest hydrolysed with HCl under reflux instead of with β-glucuronidase.

was released in ether-soluble form under these conditions was devoid of oestrogenic activity (cf. also Riegel and Mueller, 1953) β-Glucuronidase hydrolysis was relatively ineffective, in contrast to the observations on the *serum* protein-bound material. These, and other findings to be reported, suggest that the association of steroidal metabolites with liver proteins differs markedly in character from the serum protein binding phenomenon. The former may be a reflection of the detoxification function of the liver, as has previously been indicated (Roberts and Szego, 1955).

Attempts to Characterize the Enzyme System which Catalyses the Association of Steroidal Products with Serum Protein

Preliminary studies in progress in our laboratories through the efforts of Miss Janice Kayahara have indicated that the serum protein binding phenomenon will proceed in a suitably fortified rat liver homogenate. The "complete system" is indicated in Fig. 3, and it should be noted that the approach

Complete system: 0·5 ml 30 per cent homogenate rat liver (in 0·154M-KCl: 0·002M nicotinamide). Incubation medium consisting of:

- KH_2PO_4, 1·6 × 10^{-1}M
- $MgCl_2$, 2·6 × 10^{-3}M
- K_2SO_4, 1·6 × 10^{-2}M
- $KHCO_3$, 1·1 × 10^{-3}M
- Fumarate, 2·6 × 10^{-3}M
- DPN, 3·4 × 10^{-3}M

FIG. 3 Provisional requirements for enzymic binding of [16-^{14}C]oestrone or its metabolites to serum proteins *in vitro*

being undertaken will be to remove ingredients systematically from this too-complex medium. In Fig 4, which depicts first efforts in this direction, it may be noted that substitution of TPN for all or part of the DPN reduces the efficiency of the

of the transport of endogenous circulating oestrogens or their metabolites in the rat, or in other species (cf. Roberts and Szego, 1946).

In the course of these studies it became evident that a

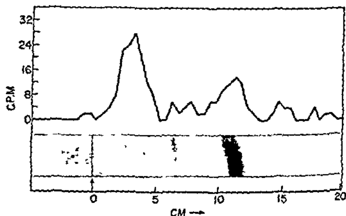


FIG. 2. The occurrence of albumin-bound radioactivity after

acetate, 0 029M, sodium chloride, 0 011M; μ=0 08; pH adjusted to 8 6), at a constant current of 0·5 mA per cm. for 16 hours. For analysis of distribution of radioactivity, see text, and Szego and Roberts (1956).

significant proportion of radioactivity derived from ^{14}C-labelled oestrogen became firmly bound to the liver residue, and that this association differed in several respects from that observed to occur with the serum proteins of the incubation

indications of inhibition by heavy metals, in contrast to lack thereof in the isolated glucuronosyl transferase system (Isselbacher, 1956), suggests that activity of the latter is not sufficient to account for the complete phenomenon in our system, however.

Fig. 5 reveals that the association of the bulk of the bound radioactivity in the homogenate system occurs with the albumin fraction of the medium when the latter is subjected

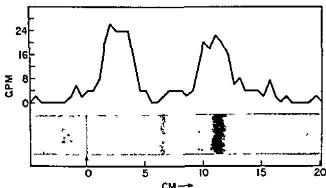
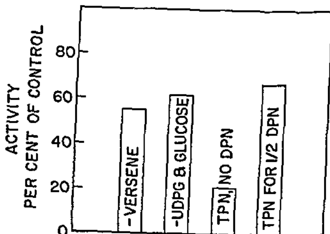


FIG. 5. Occurrence of protein-bound radioactivity after the incubation of [16-^{14}C]oestrone in the presence of rat liver homogenate in a synthetic medium. Conditions of medium composition and of incubation were as described for homogenate control in Fig. 4. Electrophoretic analysis as in Fig 2.

to paper electrophoretic analysis, an observation quite analogous to that in the mince preparation earlier discussed. The significance of a considerable degree of radioactivity in the α_1-globulin region of Fig. 5 requires further study.

In preliminary efforts towards purification of the enzyme, a liver acetone powder and its buffer extract have been shown to contain enzymic activity, but at a level sharply reduced from that of the whole homogenate (Fig. 6). In none of the broken cell or cell-free preparations observed to date has the activity been quite as high as that of the mince control for this series (Fig. 6). However, *in a single observation made too late* for inclusion in the figure, it was noted that a 15 per cent

system. Heavy metals may inhibit, as inferred in part from the gain in efficiency in the presence of Versene. This is in keeping with earlier observations on the liver mince which had indicated profound inhibition in the presence of trace amounts of mercury. Removal of uridine diphosphate glucose (UDPG)



and glucose also curtailed the activity of the homogenate, a finding which is suggestive of failure of glucuronide synthesis (cf. Isselbacher, 1956), and thus, conjugation, preliminary to association of the steroidal metabolites with serum protein in the medium (cf. Roberts and Szego, 1946, 1953, 1955; Szego, 1953, 1955; Szego and Roberts, 1953). The apparent requirement for DPN may indeed be related to this process. The

be more fruitfully studied in context with physiological observations, particularly since these forms appear to require metabolic intervention for their biosynthesis. Hence it seemed advisable to determine whether the *in vitro* system would lend itself to the analysis of protein association with steroids other than the oestrogens, whether pairs of hormonally active steroids would interact in this system, and further, whether the results achieved in these directions were in harmony with observations made *in vivo*.

It will be recalled that a series of papers from these laboratories had described the profoundly inhibitory effect of concomitantly administered ACTH (Szego and Roberts, 1948) or adrenocortical steroids of specific configuration (Szego, 1952; Szego and Roberts, 1953), upon oestrogen-induced growth and metabolic responses in the uterus of the ovariectomized rat (cf. also Talalay *et al.*, 1952). A portion of these findings has received confirmation (Velardo and Sturgis, 1956). The suggestion had been advanced in the course of interpretation of these data (Szego, 1952; Szego and Roberts, 1953; Roberts and Szego, 1953) that the oestrogens and certain adrenocortical steroids of appropriate structure compete for active sites on protein molecules essential for steroid activity, either as transport complexes apparently formed in the liver, or at the target cell itself. This suggestion was based upon the following evidence:

1. The apparently specific structural requirements for the antagonist;
2. The "titrability" of its influence, from mild inhibition to complete obliteration of the response to oestrogen, in accordance with dosage;
3. The lack of discernible effect of the antagonist alone upon the metabolic processes which were influenced by the oestrogens, even though blockade of oestrogenic stimulation could be achieved; and, finally,
4. The demonstrated affinity of certain of the steroid hormones for protein.

homogenate exceeded the capacity of both that of the 30 per cent homogenate and of the mince, all reaction rates having been expressed on an equivalent nitrogen basis.

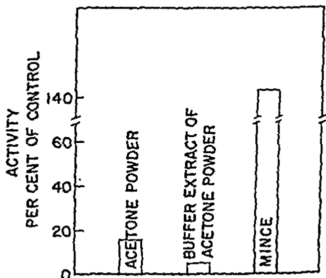


FIG. 6. Influence of type of liver preparation on enzymic binding of [16-^{14}C]oestrone or its metabolites to serum protein. Control (100 per cent) activity established for 30 per cent homogenate in the complete medium (cf. Fig. 4). Medium for the mince, Krebs

Steroid Interaction in the *In Vitro* System

Some of the methods described above have proved useful for the analysis of steroid interactions. As mentioned at the outset of this discussion, transport forms of the steroid hormones, though of great intrinsic interest, would appear to

Hepatic Intervention in the Binding of Cortisol and Corticosterone or their Metabolites to Serum Proteins *In Vitro*

Fig. 7 demonstrates the electrophoretic pattern and distribution of radioactivity of the medium following incubation of [4-^{14}C]cortisol in serum alone. It will be noted that except for

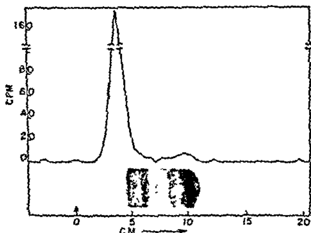


FIG. 7. Absence of significant protein-bound radioactivity after incubation of 0 035 μmoles [4-^{14}C]cortisol in 2 ml. rat serum alone. Incubation carried out under a constant stream of 95 per cent O_2–5 per cent CO_2; otherwise as in legend to Fig. 2 and in Szego and Roberts (1956). Electrophoresis of 20 μl. of resultant medium conducted as in Fig 2.

a trace of radioactivity in the albumin area, the bulk is unbound and occurs behind the slowest of the proteins (cf. also Westphal, Firschein and Pearce, 1955). Except for the trace of activity in the albumin area which occurs with [4-^{14}C]cortisol without hepatic participation, these findings are quite analogous to those with isotopically-labelled oestrogen control in the presence of serum alone (cf. Szego and Roberts, 1955*a*, 1956).

When incubation is carried out in the presence of surviving liver, moreover, radioactivity derived from [4-^{14}C]cortisol is

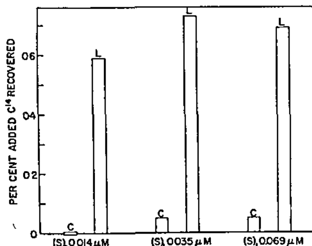
Although these qualitative and semi-quantitative observations lent considerable support to the hypothesis, it appeared highly desirable to test its validity with more demanding criteria, including, as far as possible, those customarily applied in classical enzymology.

Accordingly, studies were undertaken in the *in vitro* system described earlier. Since these experiments antedated the homogenate studies previously mentioned [and indeed, have been reported in part (Szego and Roberts, 1956), and in preliminary form (Szego and Roberts, 1955*a*, *b*)], the serum medium-liver mince preparation was employed in the presence of non-isotopic and ^{14}C-labelled steroids,* singly and in pairs as indicated. Simultaneous analysis of the medium and liver residue by the cold acetone fractionation scheme, and of the former by electrophoresis was carried out, together with determination of distribution of radioactivity as previously described (Szego, 1953; Szego and Roberts, 1956).

It would appear that the requirements for classification as competitive of the type of inhibition exerted by the 11-oxy-17-hydroxycorticosteroids upon oestrogen action *in vivo*, if it were observed to extend to *in vitro* inhibition of binding to protein, would be as follows:

1. The obligatory rôle of a liver enzyme system for significant protein complex formation of the corticosteroid moiety.
2. Association of the corticosteroid moiety with the same protein fraction as that which serves as carrier for oestrogen or its metabolites (herein, one which migrates with the mobility of serum albumin).
3. Conformation to the Lineweaver-Burk and similar analyses of interaction when varying amounts of inhibitor are present at different levels of substrate.

These three criteria have been applied to this problem with the following results.



procedure outlined in Fig. 1

Attempts to Characterize the Pattern of Inhibition by Cortical Steroids of Oestroprotein Formation *In Vitro*

Notwithstanding the crudity of the system, it will be demonstrated that when the classical criteria are applied for testing the nature of the antagonism exerted by cortisol on oestroprotein formation *in vitro*, the pattern of inhibition appears to be a competitive one.

Incubation of the primary substrate, [16-^{14}C]oestrone, in serum was carried out at three concentration levels with and without inhibitor, unlabelled cortisol*, in the presence of minced liver for 2 hours. Previous studies had established

* Cortisol was kindly made available by Dr Elmer Alpert of Merck-Sharp and Dohme, Inc.

associated to a striking degree with the albumin area, as depicted in Fig. 8. Thus, the same protein carrier is involved as in the case of hepatically-catalysed oestroprotein formation *in vitro*.

The quantitative importance of hepatic intervention in this process is illustrated in Fig. 9, in which are seen the relative proportions of ^{14}C associated with serum proteins of the

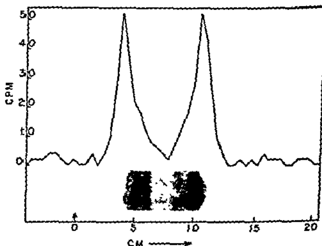


FIG. 8. The presence of albumin-bound radioactivity after incubation of [4-^{14}C]cortisol in the presence of 250 mg. surviving rat liver in an homologous serum medium Experimental conditions as in Fig 7 and Szego and Roberts (1956)

medium in a form labile to β-glucuronidase hydrolysis, with and without liver tissue present during the incubation of varying concentrations of [4-^{14}C]cortisol. Analysis of the medium was carried out by a modified cold acetone fractionation method in this instance. It is clear from Fig. 9 that the presence of the liver enzyme system is obligatory for significant protein complex formation *in vitro* with [4-^{14}C]cortisol. Isotopically-labelled corticosterone behaved similarly in parallel experiments.

Thus it appears that the first two of the criteria indicated above have been satisfied.

which is associated with the liver protein residue, a dichotomy to which attention was earlier directed (Szego, 1953; Roberts and Szego, 1955).

A related line of investigation being pursued by Talalay and

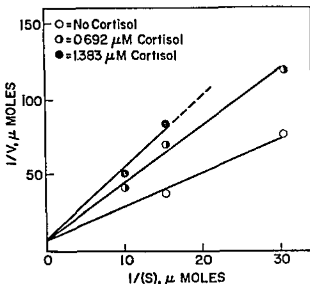


FIG. 10 Pattern of inhibition by cortisol of oestroprotein biosynthe-

present in 1 ml. aliquot of original (2 ml.) incubation medium Fractionation of the medium was carried out as in Fig 1, on a 1 ml.

Marcus (1956) has revealed profound inhibitory effects of natural and synthetic oestrogens upon the activity of a highly purified bacterial β-hydroxysteroid dehydrogenase in the oxidation of testosterone. In the course of these elegant studies, in which considerable emphasis has been placed upon utilization of the enzyme as a model protein surface for the

that the velocity of oestroprotein formation was linear up to that time, and since the radioactivity measurements gained in precision as greater concentrations of protein-bound material accumulated, this moderately long incubation period was chosen. The results from analysis of the protein fractions derived from the cold acetone fractionation procedure are depicted, although similar data were obtained from the analysis of the medium by the electrophoretic resolution method followed by determination of isotope and protein distribution.

Fig. 10 reveals the results of such an experiment when the data are subjected to the double-reciprocal plot of Lineweaver and Burk (1934). It may be noted from Fig. 10 that while the intercept remains unchanged, the slope is dependent upon *both* substrate concentration and inhibitor concentration. Thus, the data conform to the requirements for the demonstration of competitive inhibition. Further support for this statement was obtained from the observation that the per cent inhibition is dependent upon relative concentrations of substrate and inhibitor, being greater at low than at high oestrogen concentrations. Additional evidence suggestive of competition is the significant dependence of slope (v/v_i : [I]) upon substrate concentration in the graphical analysis of Ebersole, Guttentag and Wilson (1944).

Preliminary data of a similar nature have been obtained in the reciprocal experiment using [4-^{14}C]cortisol as primary substrate, with and without the presence of unlabelled oestrone or oestriol.

In contrast to the apparently competitive nature of steroidal interaction in the biosynthesis of transport forms of these substances *in vitro*, the process involved in binding of steroid metabolites to the liver proteins does not appear to be similarly regulated. While some inhibition is seen in the presence of the "antagonist", its relative independence of substrate concentration is notable. This is believed to constitute further evidence of the distinction between the so-called transport form, that is the *serum* protein-bound material, and that

extended without the availability of more generally labelled steroid of infinitely higher specific activity which would permit the administration of truly physiological, and thus more meaningful, levels of steroid.

Another problem which has arisen involves the specificity of the antagonistic interaction among steroids in the *in vitro* system. It has been observed that desoxycorticosterone inhibits oestroprotein formation from [16-^{14}C]oestradiol *in vitro*, whereas in our hands it does not appreciably antagonize oestrogenic stimulation of uterine growth and metabolism *in vivo* at dose levels at which cortisol and cortisone block completely. This serious flaw in the unqualified interpretation of the data according to the aforementioned hypothesis may also be resolved by purification of the system and by the utilization of a broader range of "substrate" to "inhibitor" ratios. Nevertheless, it is clear that the present series must be extended to a wide variety of steroid pairs to determine the extent to which interaction *in vitro* reflects the physiological status of these substances in the whole living animal. Such a programme is in progress in our laboratories.

Notwithstanding these discrepancies, together with the acknowledged sin of oversimplification which has been indulged in, it is possible that with continued testing, a broad principle of steroid hormone action and interaction will emerge from these and related studies. There is a large and rapidly expanding literature on the blockade of the action of steroidal and similar substances by structurally related analogues. Some of the more recent examples of such interaction, which have appeared since this topic was subjected to comprehensive review (Roberts and Szego, 1953), include inhibitory effects of certain other phenolic steroids on the uterine growth response to 17β-oestradiol (Hisaw, Velardo and Goolsby, 1954) and to oestrone (Hisaw, Velardo and Goolsby 1954; Huggins and Jensen, 1955). Specific antagonism between pairs of steroids at levels other than the reproductive tract is not uncommon, namely, the observations of Woodbury and Sayers (1950), of Selye and colleagues (Selye, 1950,

investigation of steroidal interactions, analysis of the pattern of inhibition revealed that it conformed neither to competitive nor non-competitive substrate : inhibitor interaction.

Considering the provisional nature of the present experiments from a quantitative viewpoint, it is encouraging to note that the data obtained in the *in vitro* system are in harmony with the antagonistic interactions exhibited by certain cortical steroids and the oestrogens *in vivo*, and are thus compatible with the working hypothesis that these hormones compete *for reactive sites on protein molecules essential for steroid* transport or activity, as earlier advanced from these laboratories (*vide supra*).

Let me be the first to point out, however, the occurrence of several flies in this ointment. Most of these derive from the difficulties inherent in working with such a crude preparation. The order of magnitude of the provisionally calculated K_m of oestrone ($2 \cdot 36 \times 10^{-4}$ M. $\pm$ S.E. $0 \cdot 19 \times 10^{-4}$ in four experiments) is not strikingly different from that determined for cortisol under similar circumstances. Yet the relative concentration requirements for significant inhibition by cortisol of oestroprotein binding are rather high, in keeping with the *in vivo* observations. Since the overall reaction in the *in vitro* "model" system appears obviously composed of more than one process, this problem must await resolution through purification and isolation of the stepwise reactions involved. It must also be recognized that the relatively unphysiological concentrations of steroid in the *in vitro* system may lead to artifacts of complex formation with a protein that appears to be utilized primarily for bulk transport. That this is probably the case is suggested by data derived from an experiment in which a massive intravenous dose of [16-^{14}C] oestrone was administered to a Sprague-Dawley female rat. Serum withdrawn after one minute was subjected to electrophoretic separation and radioisotopic analysis. It was observed that while albumin still occupied the rôle of principal carrier of bound radioactivity, some distribution to other proteins was indicated. The *in vivo* experiments cannot be profitably

... In Symposium on Atherosclerosis, p. 513 Wash-
...Research Council.
(1954). *J. clin.*
..., 102, 335.
...s, 12, 134.
...ISTER, L. (1956).
... *J. Amer. Chem. Soc.*, 56, 658.
... *Biol., N.Y.*, 78, 143.
...). *J. biol. Chem*, 210, 249.
...ALL, J E. (1955). *J. biol. Chem.*,
212, 405.
... *Endocrinology*, 39, 183.
... *l. Rev.*, 33, 593.
... *Rev Biochem.*, 24, 543.
... *Quart. J. exp. Physiol*,
38, 233.

SELYE, H. (1950). *In* The Physiology and Pathology of Exposure to Stress. Montreal: Acta, Inc.

SELYE, H. (1955). *Science*, 121, 368.

SELYE, H, and BOIS, P. (1956). *Proc Soc. exp. Biol., N.Y.*, 92, 362.

SIPERSTEIN, M. D., NICHOLS, C. W. JR, and CHAIKOFF, I. L. (1953). *Circulation*, 7, 37.

SZEGO, C. M. (1952). *Endocrinology*, 50, 429.

SZEGO, C. M. (1953). *Endocrinology*, 52, 669.

SZEGO, C. M. (1955). *Endocrinology*, 57, 541.

SZEGO, C. M. (1956). *In* Symposium on Physiological Triggers, ed. Bullock, T. H., p. 152. Washington: American Physiological Society.

SZEGO, C. M., and ROBERTS, S. (1946). *Proc. Soc. exp. Biol., N.Y.*, 61, 161.

SZEGO, C. M., and ROBERTS, S. (1948) *Amer. J Physiol.*, 152, 131.

SZEGO, C. M., and ROBERTS, S. (1953). *Recent Progr. Hormone Res*, 8, 419.

...10, 4...

WESTPHAL, U., FIRSCHEIN, H. E, and PEARCE, E. M. (1955). *Science*, 121, 601.

WOODBURY, D. M., and SAYERS, G. (1950). *Proc. Soc. exp Biol, N.Y.*, 75, 398.

WOOLLEY, D. W. (1946). *J. biol. Chem.*, 164, 11.

1955; Selye and Bois, 1956), and of Gardner, Berman and Deane (1954), to cite just a few examples. The appreciable reduction in plasma cholesterol levels under certain conditions of administration of phytosterols (Peterson, 1951) and of dihydrocholesterol (Siperstein, Nichols Jr. and Chaikoff, 1953), may be indicative of a related phenomenon (cf. also Gould, 1954). The apparent behaviour as antimetabolites of certain partially hydrogenated derivatives of carcinogenic polycyclic hydrocarbons has been suggested (cf. Kotin *et al.*, 1956). Although the evidence is accumulating from such *in vivo* observations, it falls, on theoretical grounds, far short of proof for strictly competitive phenomena.

The line of attack employed in the present investigation may well be profitably applied to the solution of the precise nature of the antagonism observed in some of these and other examples of steroidal interaction. The provisional interpretations arising from the present studies suggest that the nature of the inhibition is competitive in the instances cited. If these observations are confirmed and extended it is manifestly clear that a principle of the broadest implications in the field of steroidal, and perhaps other hormonal action (cf. Woolley, 1946; Frieden and Winzler, 1949), may emerge.

Acknowledgements

Valuable technical assistance in the course of these studies was contributed by Miss Dorothy Wolcott, Mr. Edwin M. Weller, and Mr. John E. Creange. Dr. John E. Snoke and Dr. Irving Zabin of the Department of Physiological Chemistry, University of California, Los Angeles, kindly made many helpful suggestions during discussion of a portion of the data.

REFERENCES

ANFINSEN, C. B. (1954). *In* Symposium on Atherosclerosis, p. 217. Washington: National Academy of Sciences—National Research Council.

EBERSOLE, E. R., GUTTENTAG, C., and WILSON, P. W. (1944). *Arch. Biochem.*, 3, 399.

FRIEDEN, E., and WINZLER, R. J. (1949). *J. biol. Chem.*, 179, 423.

GARDNER, L. I., BERMAN, H., and DEANE, H. W. (1954). *Endocrinology*, 55, 417.

Migeon: We have observed a marked disturbance of cortisol metabo-

Szego: At that level of course, competition may be in terms of transport protein following saturation of a specific carrier.

Roberts: I should think that it is unlikely that the competition would

Roberts: But, consider the relatively enormous amount of glucuronides that are formed during pregnancy!

Samuels: This depends on how you consider the mass of the tissue, and the time involved, and the question of whether you have ideal conditions

β-glucuronidase.

Pearlman: In this incubation process, is there any evidence that

end of a run of the usual duration. In other words, there are metabolites which are fragments of the original substrate, but still radioactive and move ahead even of the protein-bound material

Samuels. Have you identified the material in the albumin fraction after incubation with cortisol as cortisol by paper chromatography?

Szego: No.

DISCUSSION

Astwood: When one thinks of various clinical states, one does not recall one that supports the hypothesis that cortical steroids inhibit oestrogen Has the *in vivo* inhibition been studied on other criteria besides this

concomitant gonadotrophin alterations or evidence of ovarian failure.

Astwood: Have you tried other types of oestrogen assay?

Szego: It was not just the early effects of the oestrogen in the first 4

to

of

with albumin after incubation with radioactive cortisol? Is it a metabolite, or cortisol, or is it a glucuronide?

Szego: β-Glucuronidase treatment of the partially digested serum proteins prepared by the cold acetone method does release substantial amounts of ether-soluble radioactivity whether isotopic cortisol or oestrogen is used as substrate. We have not, however, eluted the cortisol moiety from the albumin area of the strip as we have the oestrogen metabolites. In the case of the oestrogens such material retains at least

adis-

This

ydro-

lysis, and no longer oestrogenic.

Bush: It would affect your interpretation quite considerably?

Szego: Very much so. I have no doubt that it is very unlikely that it would be the original unchanged substrate.

curonide.

Samuels: Have you given that treatment to the cortisol ...

Szego: Yes.

THE USE OF [16-^{3}H]ALDOSTERONE IN STUDIES ON HUMAN PERIPHERAL BLOOD

P. J. Ayres, O. Garrod, Sylvia A. S. Tait,* J. F. Tait* and G. Walker

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and W. H. Pearlman

Department of Biochemistry, Guy's Hospital, London

Previous speakers (Pearlman, 1957; Samuels, 1957) have described how the study of the metabolism of radioactive steroids may contribute to our knowledge of the amounts and physicochemical state of the hormones and their metabolites in human peripheral blood. They have shown that quantitative data concerning the steroid, such as rate of production, rate of turnover in the blood and miscible pool, may be obtained by following the fate of injected radioactive hormone. This information may help to explain the amounts of free hormone in blood.

Of the three most important hormones of the human adrenal cortex capable of maintaining life, cortisol (Hellman *et al.*, 1954; Peterson *et al.*, 1955; Peterson and Wyngaarden, 1956; Migeon *et al.*, 1956*a*) and to a lesser extent corticosterone (Migeon *et al.*, 1956*b*) have been studied in this way, but metabolic studies with aldosterone have not previously been reported. Similarly, although there is reasonable agreement as to the concentrations of cortisol in human peripheral blood (Bliss *et al.*, 1953; Bush and Sandberg, 1953; Sweat *et al.*, 1953; Morris and Williams, 1953), the corresponding values for corticosterone and aldosterone are still uncertain. This is partly due to the conflicting needs for known recovery together with high specificity in the analysis of very small amounts of

* External Scientific Staff, M R C.

Diczfalusy: Have you any evidence indicating that this protein-bound oestrogen which you obtained following glucuronidase hydrolysis is still oestrone?

Szego: No, as I mentioned earlier we have evidence only that it has not entirely lost its oestrogenic character, as indicated by the Astwood bioassay method, using the immature rat uterus and measuring water uptake in 6 hours.

Diczfalusy: In which method oestriol is much more potent than oestrone?

Szego: Yes

Diczfalusy: Is there any indication of oestriol occurring in the rat?

Szego: I have no evidence of that.

Pearlman: As far as I know oestriol is unique to the human species and possibly to the primates.

Diczfalusy: What about the isolation of oestriol from an extract of female willow catkins by Skarzymski (1933. *Nature, Lond.*, **131**, 766)?

Pearlman. I am not sure that that is relevant to the physiology of human reproduction!

Szego: Are you ruling out the observations of Axelrod on dog bile?

Pearlman: I am not altogether convinced of that.

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* External Scientific Staff, M.R.C.

steroid, a conflict which can be resolved by adding radioactive steroid to the blood *in vitro* before analysis. The radioactivity can then be used to indicate losses in the extensive isolation procedures necessary to ensure specificity. For both *in vivo* metabolic and *in vitro* analytical uses of the radioactive steroids, it is essential to add amounts much smaller than are already present in the body or the collected blood but sufficiently radioactive to be followed with ease through any process. We have prepared [16-^{3}H]aldosterone and corticosterone of high specific activity ($\simeq$ 1 μc per μg.) for this purpose and have developed a simple and sensitive method for assaying samples containing tritium. These tritiated steroids have been applied to preliminary analytical and metabolic studies.

Preparation of tritiated adrenal steroids

Fifteen mc [16-^{3}H]progesterone (specific activity 1·7 μc per μg.), synthesized by one of us (Pearlman, 1956), were incubated with 20 g. capsule strippings of ox adrenal glands under conditions described in detail elsewhere (Ayres, Simpson

Table I

Steroid	*Amount in μg*	*Specific Activity μc per μg*	*% yield radioactivity*
Progesterone	2,800	1 39	39
Corticosterone acetate free	1,940	1 39 1 37	27
Aldosterone diacetate free	238	0 86 0·90	2 1
Cortisol acetate	35	0 58	0 2

Data on steroid produced by incubating 9 mg. [16-^{3}H]progesterone (specific activity 1·74 μc per μg.) with 20 g. capsule strippings of ox adrenal gland. Values corrected approximately for recovery.

and Tait, 1956). The yields of the steroids produced and their specific activities are shown in Table I. These include corticosterone and aldosterone of suitable specific activity, 1·4 and 0·9 μc per μg. respectively.

Isolation procedures and measurements of specific activities

The values for corticosterone, aldosterone and cortisol were obtained in the following manner. The extract from the incubation, after preliminary purification on a silica gel column (Bush and Sandberg, 1953), was chromatographed on several partition columns, which had a resolution of about 600 theoretical plates for these steroids. The first column (top, Fig. 1) separated corticosterone from a mixture of cortisol and aldosterone, both of which ran at the same speed with this solvent system but were completely separated from one another on a second column (middle, Fig. 1). The corticosterone was also further purified on another column (bottom, Fig. 1). The three steroids were then separately acetylated and the acetyl derivatives purified on three further columns. Portions of the eluted fractions from these columns were assayed for tritium. The amounts of steroids in the fractions were estimated by measuring their soda fluorescence compared with that of standards, by a fluorimeter following paper chromatography (Ayres *et al.*, 1957). The specific activity of a particular steroid acetate in those fractions of the final column containing appreciable radioactivity could thus be measured and compared. They were found to be equal within the limits of experimental error. The specific activities of the acetyl derivatives recorded in Table I were therefore measured after pooling these fractions. Corticosterone monoacetate and aldosterone diacetate were then hydrolysed and the free compounds purified by chromatography on two columns as described previously (Fig. 1). The specific activities of the free compounds were then estimated again by counting and measurement of soda fluorescence. For a particular steroid, the specific activities of the free compound and its acetyl

steroid, a conflict which can be resolved by *adding radio-*active steroid to the blood *in vitro* before analysis. The radioactivity can then be used to indicate losses in the extensive isolation procedures *necessary to ensure specificity*. For both *in vivo* metabolic and *in vitro* analytical uses of the radioactive steroids, it is essential to add amounts much smaller *than are already present* in the body or the collected blood but sufficiently radioactive to be followed with ease through any process. We have prepared [16-^{3}H]aldosterone and corticosterone of high specific activity ($\simeq$ 1 μc per μg.) for this purpose and have developed a simple and sensitive method for assaying samples containing tritium. These tritiated steroids have been applied to preliminary analytical and metabolic studies.

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Table I

Steroid	*Amount in μg.*	*Specific Activity μc per μg*	*% yield radioactivity*
Progesterone	2,800	1·39	39
Corticosterone			
acetate		1·39	
free	1,010	1·37	27
Aldosterone			
diacetate		0·86	
free	238	0·90	2·1
Cortisol			
acetate	35	0·58	0·2

specific activity was measured as for the biosynthetic material. Isolation of the steroids was also carried out as previously described, but it was found possible to determine the specific activity of the corticosterone on the final column of the free compound (bottom, Fig. 1), and formation of the monoacetate was unnecessary. Separation of the cortisol and aldosterone before acetylation was also not required. The cortisol monoacetate and aldosterone diacetate, obtained by acetylating the mixture of the free compounds on the first column (top, Fig. 1), were separated on a final column in which the mobile phase was changed after the appearance of aldosterone diacetate in the eluate. This is the procedure which has been used for the analysis of urine (Ayres *et al.*, 1957) except that then [4-^{14}C]cortisol and corticosterone and [carboxy-^{14}C]aldosterone diacetate were used as indicators. The specific activities of the ^{14}C-labelled corticosterone and aldosterone were, however, too low for their use as indicators in blood, and tritiated steroids were substituted.

Following injection of tritiated aldosterone, the daily rate of production of the hormone was estimated by hydrolysing an immediate 24-hour collection of urine by continuous extraction at pH 1 and room temperature and determining the specific activity (^{3}H activity/amount of steroid) of the aldosterone released. Small amounts of [carboxy-^{14}C]aldosterone diacetate were also added to the extract before the final column so that our usual analytical procedure could be carried out (Ayres *et al.*, 1957) and the correlation of ^{14}C, ^{3}H and the steroid on the final column investigated. In all cases, where values are quoted, good correlation was observed.

The radioactivity of the tritiated material was assayed by depositing the sample from a chloroform solution on a circular planchet, 3 cm. in diameter, which had a large number of circular concentric grooves, about 0·05 mm. deep, scored on its surface. This ensured uniform distribution of the radioactivity. If the sample weighed less than 30 µg., it was dissolved in 0·3 ml. of a solution of wax in chloroform (800 µg. beeswax per ml.) and 0·2 ml. of the resulting solution was

derivative were found to be equal on a molar basis. The radiochemical purity of these final preparations was confirmed for aldosterone by bioassay and recrystallization of a portion

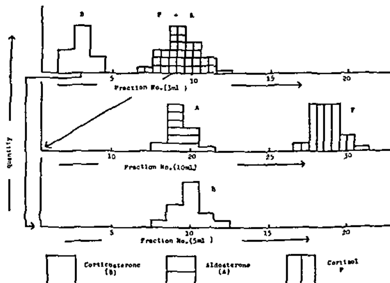


FIG. 1. Partition columns for purification of free compounds

Columns :—

60 cm. in length, 1 cm. internal diameter;
24 g. Celite 545; 12 ml. stationary phase.

Solvent systems :—

Top column
Methanol : water (1 : 1 v/v);
benzene : ethyl acetate (16 : 1. v/v)

Middle column
Methanol : water (1 : 1. v/v);
benzene : petroleum ether. b.p. 80–100° (7 : 3. v/v).

Bottom column
Methanol : water (4 : 1. v/v);
benzene : petroleum ether. b p. 80–100° (17 : 3. v/v)

from added pure compound, and by recrystallization of the monoacetate in the case of corticosterone.

For analytical purposes, very small amounts of tritiated corticosterone and aldosterone, together with [4-^{14}C]cortisol, were added to the blood and, after purification, the final

24 hours following precipitation of proteins. This fractionation was similar to that employed by Migeon and co-workers after injection of [4-^{14}C]cortisol and corticosterone except that, compared to the procedure adopted by those workers, we used more chloroform for the first extraction, more enzyme

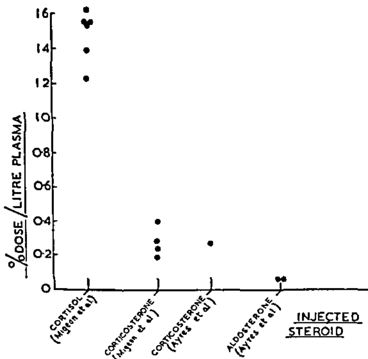


FIG 2 Chloroform-extracted radioactivity, pH 6, as per cent dose/l. plasma measured 2 hours after injection of various radioactive steroids.

for the second, and chloroform instead of ether for the third extraction at pH 1. Also, all extracts were purified before assay by silica gel chromatography as described by Bush and Sandberg (1953).

The results for the free steroid fraction (i) in plasma obtained 2 hours after injection are shown in Fig. 2. Corresponding values, obtained by Migeon and collaborators (1956*a*, *b*) 2

placed on the planchet. Chloroform was added to spread the solution over the entire surface. The planchet was then counted in a Tracerlab flow counter. The overall efficiency was about 12 per cent and the coefficient of variation, neglecting statistical errors, about 2 per cent. If the sample were heavier, it was deposited directly on the planchet, and after the initial count, a self-absorption correction was carried out by adding a standard amount of tritiated steroid of negligible weight and recounting the sample. The coefficient of variation, without counting errors, was about 5 per cent.

Stability of the label

As a result of the experiment with [16-^{3}H] and [4-^{14}C] progesterone (Pearlman, 1957) there seems no reason to doubt the stability of the label at the 16-position, unless transformation of the molecule occurs near the point of attachment of the tritium atom. We have also obtained good quantitative agreement for the radioactivity in various fractions of blood as between our results after giving [16-^{3}H] corticosterone and those of Migeon and co-workers after [4-^{14}C]corticosterone. Radioactivity might be lost if the side-chain were removed, or even perhaps if 17-hydroxylation occurred, but there is no evidence to indicate that these are major reactions in the metabolism of steroids.

Disappearance of [16-^{3}H]aldosterone from human blood

Two normal young males received intravenously 2 and 6 µg. [16-^{3}H]aldosterone respectively at 9 a.m. Blood was collected from both subjects two hours later at 11 a.m., and also from one subject at 12 noon. The radioactivity in the plasma was measured following: (i) chloroform extraction (4 × 1·5 vol.) at pH 6, (ii) chloroform extraction (4 × 1·5 vol.) after incubation with β-glucuronidase (1000 u. "ketodase" per ml. at pH 4·5 and 47°C for 15 hours) following precipitation of the proteins by alcohol, (iii) continuous chloroform extraction at room temperature and pH 1 for

quantities of blood necessary for analysis. Fig. 3 shows the radioactivity in the free steroid fraction of plasma for one subject, 2 and 3 hours after giving tritiated aldosterone, compared with values obtained at different times after giving

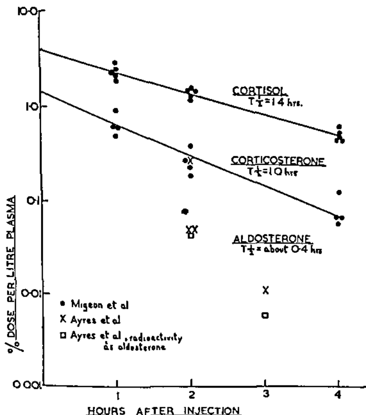


FIG. 3. Chloroform-extracted radioactivity, pH 6, per cent dose per l. plasma, measured 1, 2, 3 and 4 hours after injection of various radioactive steroids.

labelled cortisol and corticosterone. For aldosterone, the radioactivity is reduced sixfold during the period from 2 to 3 hours after the injection, compared to 1·6-fold in the corresponding period for cortisol and 2·2-fold for corticosterone. The half-lives would therefore be 0·4, 1·4 and

hours after giving [4-^{14}C]cortisol and corticosterone, and after [16-^{3}H]corticosterone, are also shown. After giving [16-^{3}H] aldosterone, the radioactivity in the free steroid fraction (0·060 and 0·059 per cent of dose per l. plasma) is much less than the corresponding results after [4-^{14}C]cortisol (mean 1·47, range 1·23–1·63 per cent/l., 5 cases) or [4-^{14}C]corticosterone (mean 0·28, range 0·19–0·40 per cent/l., 4 cases) or [16-^{3}H]corticosterone, 0·27 per cent/l. Although the data is still limited, it would appear, even at this stage of the investigation, that the amount of radioactivity in this fraction after giving labelled aldosterone is very low relative to that obtained after giving the other two adrenal hormones.

The corresponding figures obtained by Peterson and Wyngaarden (1956) after giving [4-^{14}C]cortisol are even higher than those of Migeon and collaborators (1956*a*) with the same steroid, although the former workers measured the radioactivity specifically as cortisol. Migeon and co-workers claim that about one half of the radioactivity in their free steroid fraction is due to cortisol. After giving [16-^{3}H]aldosterone, about 80 per cent of the total radioactivity in the free steroid fraction in the plasma 2 hours after injection was due to aldosterone itself. Thus it seems that 2 hours after the injection of the two hormones, radioactivity in the blood due to the administered steroid is about 20 to 30 times less after aldosterone than after cortisol. The lower amount of radioactivity at this time may be due to a relatively greater miscible volume, that is a greater fall in radioactivity during the initial mixing period, or a three to five times shorter half-life of the activity after equilibrium has been established. The half-life of aldosterone would thus be 0·3–0·4 hours, taking 1·4 hours for the half-life of cortisol (Migeon *et al.*, 1956*a*; Peterson and Wyngaarden, 1956). This last explanation would mean that aldosterone is more rapidly metabolized or excreted compared

been examined. Fig. 5 shows the distribution of radioactivity, compared with that of known steroids in the eluates of a partition column on which aldosterone and cortisol run at exactly the same speed. As the major peak of radioactivity, which accounted for 46 per cent of the total counts in this

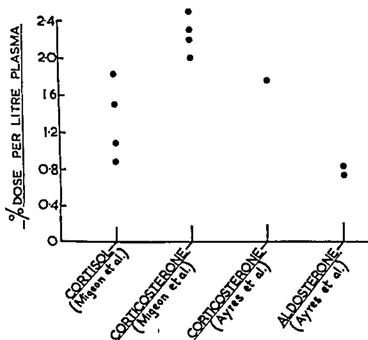


FIG 4 Radioactivity released by β-glucuronidase, per cent dose/l. plasma, 2 hours after injection of various radioactive steroids

particular fraction of one blood sample, is indistinguishable in running properties from tetrahydrocortisol, 3α : 11β : 17α · 21-tetrahydroxypregnan-20one, it seems likely that it is due to tetrahydroaldosterone, 3α : 11β : 21-trihydroxy-20-oxo-pregnan-18-ol. Work is proceeding, in collaboration with Dr. J. Barlow and Dr. A. E Kellie, on the more certain characterization of this compound. In the other blood examined, this peak *accounted for 23 per cent of the total counts in the*

1·0 hours. If the radioactivity is measured specifically as aldosterone, the half-life would appear to be even shorter, about 0·3 hours. These results indicate that more rapid metabolism or excretion of aldosterone compared with cortisol is the explanation.

The following explanations of this more rapid clearance of the aldosterone have been considered:

(*a*) Increased renal clearance of the free steroid This seems unlikely for, as is also the case with cortisol and corticosterone, the amount of free compound in the urine is small and so is the radioactivity (about 0·1 per cent of the dose) in this form after giving labelled aldosterone.

(*b*) Increased conversion to other free compounds without conjugation. This again seems unlikely, as the total radioactivity in the free steroid fraction in both blood and urine is small.

(*c*) Increased conversion to other compounds which are conjugated as glucuronides, hydrolysable by β-glucuronidase but not by mild acid conditions. This route would appear to be the main mode of metabolism for cortisol and corticosterone.

Fig. 4 shows the radioactivity released by β-glucuronidase after extraction of the free steroids in plasma obtained 2 hours after giving labelled aldosterone, with the corresponding values after radioactive cortisol and corticosterone administration. The values obtained after injecting [16-^{3}H]aldosterone are slightly lower than after giving [4-^{14}C]cortisol and [4-^{14}C] and [16-^{3}H]corticosterone. Re-incubating the plasma with fresh enzyme gave only 10 per cent more radioactivity. Increased glucuronide formation of this nature therefore seems unlikely unless the conjugate is excreted more rapidly than the corresponding metabolites of cortisol and corticosterone. However, the fraction in the urine released by β-glucuronidase contains no more radioactivity than is obtained after the administration of [4-^{14}C]cortisol.

The nature of the free steroid released by β-glucuronidase from plasma following an injection of [16-^{3}H]aldosterone has

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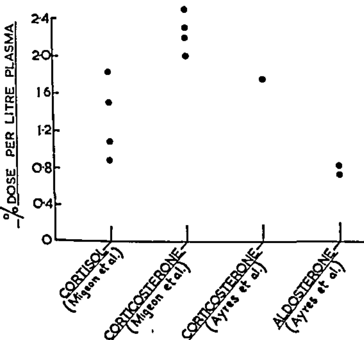


FIG. 4. Radioactivity released by β-glucuronidase, per cent dose/l plasma, 2 hours after injection of various radioactive steroids.

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[16-^{3}H]aldosterone. Whilst this value is higher than the corresponding one for [4-^{14}C]cortisol (0·05 per cent), it is much lower than those for the free steroid fraction after [4-^{14}C]cortisol and glucuronide fraction after [16-^{3}H]aldosterone. The amount of aldosterone released by this procedure was also very low and probably less than that released at neutral pH. It might also be expected that mixed conjugates of the same molecule would be formed, for example glucuronide on the 3-position and sulphate on the 18-position. However, the radioactivity in the extract obtained by continuous extraction at pH 1 and by incubating with β-glucuronidase was independent of the order in which the two procedures were carried out, which argues against the existence of large amounts of this type of conjugate in blood. This does not exclude the possibilities that the aldosterone and its metabolites, conjugated in this manner, may be more rapidly excreted than the usual glucuronides or may not be completely hydrolysed by the present methods. In a 24-hour collection of urine, about 10 per cent of the injected radioactivity is released by continuous extraction at pH 1, of which about one-third is due to aldosterone itself. These figures may have to be revised if more efficient methods of hydrolysis are developed for this type of conjugate.

(*e*) It may be that excretion of the free compound, or the conjugates of the free compound, and metabolites by the faecal route could account for the rapid clearance of aldosterone from the blood. We have no data on this point, but if it were so it would be an unusual route of excretion for a steroid by man.

Rate of Production and Plasma Values

Provided that the speed of mixing of a steroid with the miscible pool is fast compared with the rate of metabolism then

if x_A and $x_B \equiv$ concentration in plasma of any two steroids A and B,

V_A and $V_B \equiv$ miscible volumes of the steroids,

fraction, and it was also present in a similar extract from urine. Little radioactivity due to free aldosterone was released from this fraction in blood.

(*d*) Increased formation of conjugates hydrolysed by mild acid conditions. Since Luetscher's work (Luetscher and Curtis, 1955), it has been known that aldosterone is metabolized in a

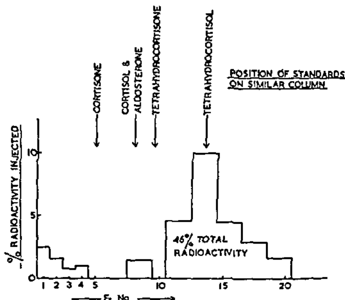


FIG. 5. Running properties of radioactivity released by β-glucuronidase from blood 2 hours after injection of [16-^{3}H] aldosterone.

different manner from cortisol. Much more free aldosterone, 20–30 times in our experience, is released from urine after continuous extraction at room temperature and pH 1 than at neutral pH, whereas the free cortisol is not increased by this procedure. It is possible that the formation of this type of conjugate, possibly a sulphate or even an easily hydrolysable glucuronide in the 18-position, could account for the rapid removal of aldosterone from the blood. Continuous chloroform extraction of blood at pH 1 and room temperature yields 0·15 per cent of the dose per l. plasma, 2 hours after giving

[16-^{3}H]aldosterone. Whilst this value is higher than the corresponding one for [4-^{14}C]cortisol (0·05 per cent), it is much lower than those for the free steroid fraction after [4-^{14}C]cortisol and glucuronide fraction after [16-^{3}H]aldosterone. The amount of aldosterone released by this procedure was also very low and probably less than that released at neutral pH. It might also be expected that mixed conjugates of the same molecule would be formed, for example glucuronide on the 3-position and sulphate on the 18-position. However, the radioactivity in the extract obtained by continuous extraction at pH 1 and by incubating with β-glucuronidase was independent of the order in which the two procedures were carried out, which argues against the existence of large amounts of this type of conjugate in blood. This does not exclude the possibilities that the aldosterone and its metabolites, conjugated in this manner, may be more rapidly excreted than the usual glucuronides or may not be completely hydrolysed by the present methods. In a 24-hour collection of urine, about 10 per cent of the injected radioactivity is released by continuous extraction at pH 1, of which about one-third is due to aldosterone itself. These figures may have to be revised if more efficient methods of hydrolysis are developed for this type of conjugate.

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V_A and $V_B \equiv$ miscible volumes of the steroids,

$T_{\frac{1}{2}}^{A}$ and $T_{\frac{1}{2}}^{B}$ ≡ half-lives of the specific activities of steroids A and B in the blood, following an injection of the respective radioactive hormones and after equilibrium has been established (usually after half an hour),

P_A and P_B ≡ rates of production of the two steroids,

then
$$\frac{x_A}{x_B} = \frac{P_A}{P_B} \times \frac{T_{\frac{1}{2}}^{A}}{T_{\frac{1}{2}}^{B}} \times \frac{V_B}{V_A}$$

If one assumes that the miscible volumes of the steroids to be considered are similar, which is probably true for cortisol and aldosterone, as indicated from Fig. 2, then

$$\frac{x_A}{x_B} = \frac{P_A}{P_B} \times \frac{T_{\frac{1}{2}}^{A}}{T_{\frac{1}{2}}^{B}}$$

The rate of production of cortisol has been deduced from measurements of the turnover rate of the miscible pool (Peterson and Wyngaarden, 1956), from clinical data on the amount necessary to maintain patients with Addison's disease (Knowlten, 1952), and from calculations derived from the quantities of metabolites in urine (Dorfman, 1954), and all agree in giving about 20 mg./day as the most likely secretion rate for normal adults. The half-life of cortisol, as defined above, is about 1·4 hours from the data of both Peterson and Migeon and their co-workers. The plasma concentration would appear to be about 10 µg. per 100 ml. plasma, this representing the mean daily value bearing in mind the diurnal variations.

The plasma concentration of aldosterone at 9.30 a.m. has previously been estimated by bioassay following chromatography in pooled samples of normal blood (Simpson and Tait, 1955) and found to be 0·02–0·20, mean value 0·16 µg. per 100 ml. plasma. The amount necessary to maintain normal electrolyte balance in Addison's disease would appear to be about 250 µg./day (Prunty *et al.*, 1954). The production rate can be calculated from the specific activity of aldosterone in the urine after administration of [16-^{3}H]aldosterone and the results for two young male students excreting normal amounts of aldosterone are shown in Table II giving values of 170 and

190 μg./day for the secretion rate. Taking the value of $T_{\frac{1}{2}}$ for this hormone to be about 0·4 hours, the mean daily concentrations in plasma, predicted by the above equations, should be about 0·03 μg. per 100 ml. plasma. This is rather lower than our previous values, but is not in conflict with those on individual subjects (determined simultaneously with the rate of production measurements on the same males) in the present studies as shown in Table II. It is unlikely that diurnal

Table II

Subject	*Rate of Production of Aldosterone μg. per day*	*24-hr. Urine Aldosterone at pH 1 in μg.*	*Blood Cortisol μg /100 ml. plasma*	*Blood Aldosterone μg /100 ml. plasma*	*Predicted Blood Aldosterone μg./100 ml. plasma*
A normal diet	170	14	10·0	0·07 0·03–0·14	0·03
C normal diet	190	8	9·8	<0·10	0·03
C low sodium diet	780	42	20	0·14 0·08–0·18	0·13

variations would alter these values, for we have observed little alteration in the urinary excretion of aldosterone during consecutive 8-hour periods. However, these measurements of aldosterone in blood cannot be regarded as being very accurate because of the low concentrations. One of the subjects was therefore depleted of sodium by sweating and placed on a low sodium diet until he was excreting only 7 m-equiv. Na per day in his urine. The urinary output of aldosterone, extracted at pH 1, rose fivefold, in agreement with the observations of other workers, and the rate of production increased in nearly the same proportion to 780 μg./day. This shows that the action of a low sodium diet is on the secretion rate rather than on the mode of metabolism. According to the above equations,

$T_{\frac{1}{2}}^{A}$ and $T_{\frac{1}{2}}^{B} \equiv$ half-lives of the specific activities of steroids A and B in the blood, following an injection of the respective radioactive hormones and after equilibrium has been established (usually after half an hour),

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the plasma values should then have been 0·12 μg. per 100 ml. plasma. The actual value was 0·14 ± 0·06 μg. per 100 ml., which is in good agreement, indicating that the lower limits of the values previously obtained on pooled plasma are more likely to be the true ones for individual specimens.

If the correct value for the aldosterone concentration in the plasma of normal subjects is about 0·03 μg. per 100 ml. then it seems doubtful whether any simple method can be evolved for its estimation in this fluid, and knowledge of the effective level of hormone reaching peripheral tissues must be deduced indirectly from the equation,

$$\text{plasma concentration} = \frac{\text{constant} \times \text{rate of production} \times T_{\frac{1}{2}}}{\text{miscible volume}}.$$

The measurement of rate of production in the particular case of aldosterone is no more difficult than obtaining values for the urinary excretion of aldosterone if the methods described in this paper are used (Ayres *et al.*, 1957), and could be easier with a few modifications. $T_{\frac{1}{2}}$ and miscible volume must ideally be obtained from the disappearance of radioactivity in plasma after injecting [16-³H]aldosterone which, although a laborious procedure, is simpler technically than measuring the aldosterone in the plasma directly. In certain circumstances alterations in the $T_{\frac{1}{2}}$ or miscible volume values would be revealed by estimating the total amount of radioactivity as aldosterone, released by extracting urine at pH 1 and also at neutral pH. This general approach to the problem would give the mean daily concentration of aldosterone in plasma, and would hence be of more value for clinical diagnosis than for acute physiological experiments.

For corticosterone, taking $T_{\frac{1}{2}}$ as 1·0 hour and the miscible volume equal to that of cortisol, then mean daily plasma concentration (μg. per 100 ml. plasma) = 0·5 × 0·7 × daily rate of production in mg.

Table III shows the plasma concentrations of cortisol and corticosterone for four normal subjects at 9.30 a.m. Measurement of the rate of production from the specific activity of the

free corticosterone in urine following an injection of [16-^{3}H] corticosterone presents considerable technical difficulties because of interfering substances on the final paper chromatogram. A more general method may be the estimation of the specific activity of an exclusive metabolite. However, in one subject, (F) (Table III), a reliable estimate of 0·84 mg. per day

Table III

Normal Subjects	*Cortisol* µg /100 *ml. plasma*	*Corticosterone* µg /100 *ml. plasma*
A	10	—
B	10	—
C	15	0 6
D	5	< 0 4
E	27	0 5
F	18	0 3
Mean	14	0 35–0 45

was obtained for the production rate, which is in fair agreement with the value obtained from the plasma concentration using the above equation. It therefore appears that corticosterone is a minor component of adrenocortical secretion in the four normal men so far investigated.

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active steroids. [illegible]
assistance of Mi [illegible]
and Mr. D. Stap [illegible]
and interest of [illegible]
Prof. J. E Ro [illegible]
Kellie and Dr. J Barlow for permission to quote results obtained in collaborative work before publication.

REFERENCES

AYRES, P. J., GARROD, O., SIMPSON, S. A. S., and TAIT, J. F. (1957). *Biochem J.* In press.

AYRES, P. J., SIMPSON, S. A. S., and TAIT, J. F. (1956). *Biochem. J.*, 63, 19P.

BLISS, E. L., SANDBERG, A. A., NELSON, D. H., and EIK-NES, K. (1953). *J. clin Invest.*, 32, 818.

BUSH, I. E., and SANDBERG, A. A. (1953). *J. biol. Chem.*, 205, 783.

[illegible]

LUETSCHER, J. A., Jr., and CURTIS, R. A. (1955). *Fed. Proc.*, 14, 746.

MIGEON, C. J., SANDBERG, A A , DECKER, H. A., SMITH, D. F., PAUL, A. C., and SAMUELS, L. T. (1956*a*). *J. clin. Endocrin. Metab.*, 16, 1137.

MIGEON, C. J., SANDBERG, A. A , PAUL, A. C., and SAMUELS, L. T. (1956*b*) *J. clin. Endocrin. Metab.*, 16, 1291.

MORRIS, C. J. O. R., and WILLIAMS, D. C. (1953) Ciba Foundation Colloquia on Endocrinology, 7, 261. London; Churchill.

PEARLMAN, W. H (1956) *Biochem. J.*, 64, 54P.

PEARLMAN, W. H (1957). This volume, p. 233.

PETERSON, R. E., WYNGAARDEN, J. B , GUERRA, S. L , BRODIE, B. B., and BUNIM, J J. (1955) *J. clin. Invest.*, 34, 1779.

PETERSON, R. E., and WYNGAARDEN, J. B. (1956). *J. clin. Invest.*, 35, 552.

PRUNTY, F. T. G., MCSWINEY, R. R., MILLS, J. H., and SMITH, M. A. (1954). *Lancet*, 2, 620

SAMUELS, L. T. (1957). This volume, p. 208.

SIMPSON, S. A , and TAIT, J. F. (1955) *Recent Progr. Hormone Res*, *11*, 183.

SWEAT, M. L., ABBOT, W. E., JEFFRIES, W. M., and BLISS, E. L. (1953). *Fed. Proc*, 12, 141.

DISCUSSION

Farrell: This is very exciting work. I am especially interested in the urinary form of aldosterone. You mentioned that about 10 per cent of the injected dose was recovered in the urine, so presumably 90 per cent of it must have been present in some other form

Tait: 10 per cent was released after continuous extraction at pH 1 and

15 per cent of the dose. The aldosterone released at pH 1 is about 5 per cent of the dose.

Farrell: So about half of the injected dose has thus far been recovered after 24 hours?

Tait: Yes.

Migeon: Don't you think that you could noticeably increase your recovery by studying the second 24-hour urine collection?

Tait. We have the urine but we haven't analysed it yet. The crude extracts might be very difficult to analyse if the radioactivity is low as there are certain technical difficulties in assays of tritium.

and intravenous injections. Now your injections were made intravenously, Dr. Tait? Intravenously injected steroid hormones are, as a rule, excreted in the urine in contrast to the intramuscularly administered hormone. In my experiments, tritiated progesterone was injected intramuscularly, and the bulk of the radioactivity was excreted in four days;

thereafter it is quite small. On the other hand, in Gallagher's experiments with intravenously injected ^{14}C-labelled progesterone, most of the radioactivity was excreted in the urine in about 8 hours or so

Migeon I agree with Dr. Pearlman that steroids given intramuscularly are excreted more slowly than those administered intravenously. However in our experiments with [16-^{14}C]oestrone, the hormone was given intravenously After the first day 37–51 per cent of the dose was excreted; the second day the cumulative excretion was 63–72 per cent, the third day 71–80 per cent. On the fourth day, 3 3 to 7 4 per cent was still excreted; on the fifth day 1 4 to 3 9 per cent; and on the sixth day 0·6 to 1·6 per cent of the dose.

Farrell Dr. Tait, in connection with the urinary aldosterone, your work has advanced this problem a very great deal, since you are able to recover 50 per cent of the injected dose, which is much more than has been previously recovered. Have you applied your techniques to, for example, patients in cardiac failure, in whom increases of the urinary aldosterone have been reported? We have heard a considerable amount of speculation to the effect that these increased values may not be due to increased production of steroid but rather to altered handling of it.

Tait: The only rate of production measurement we have made on urine from a patient with a pathological condition was on a severe but untreated nephrotic patient in which the urinary aldosterone was normal and the rate of production was raised by only 50 per cent compared with our two normal values.

Singer On the question that Dr. Farrell raised regarding altered renal handling, there are some experiments which we did in Montreal which might be of interest. We wondered whether altered renal handling might be responsible for the high output in the nephrotic syndrome. We therefore studied rats which had been rendered nephrotic and found that the aldosterone secretion in the adrenal vein blood was elevated. In one instance it was ten times higher than the level obtained in control animals. So it seems that, although there may be an alteration in renal handling in this condition, there does appear to be an increased secretion of aldosterone.

Farrell: We were interested to find in experimental cardiac failure in dogs no increased output of aldosterone into the adrenal vein. We have wondered if increased urinary excretion of aldosterone might be related to renal changes associated with cardiac disease or some other abnormality of the metabolic handling of the steroid rather than to an actual change in the rate of secretion.

Bush: How do you produce your cardiac failure?

Farrell By constriction of the pulmonary arteries

Sinard Could the increased output of aldosterone in states of what I think Dr. Venning called apprehension, such as those prior to examinations and the presentation of scientific papers, be correlated at all in the patients with cardiac failure, whom I imagine would be considerably apprehensive?

Farrell: It seems possible. The dogs were anaesthetized and presumably no emotional factors complicated the study.

Samuels: Dr. Tait, did you attempt to measure total tritium activity in the urine?

Tait: I think the technical difficulties in measuring tritium will

scintillation counter.

Singer: I have just made a quick comparison of the aldosterone values that we obtain in the adrenal vein blood of rats If one assumes that the production rate continues the same for 24 hours as it does in the 1·5 hours that we have studied, then the daily production rate, per unit

Tait: No, the only check we have on our rate of production measurements in h

intravenous

lar

Er

The other point that intrigues me is your information about the synthesis of corticosterone from your strippings. Can you tell us how much of the adrenal

Tait In the ox gland, the production of corticosterone per equal weight of

would agree with

of low

in the

be above 100 μg./day. You could expect that in pregnancy this tetra hydro-like derivative will also be excreted in relatively increased amounts.

Tait: Yes, I think that that type of urine might enable us to isolate it. It would be very nice to repeat these experiments with tritiated aldosterone in pregnancy. The answer to some of the questions raised depends on whether the mechanism of the increased urinary aldosterone in these pathological cases is the same as operates for a normal subject on a low salt diet whose rate of production is definitely increased. Our group is not yet convinced that some of these untreated pathological cases do show increased urinary aldosterone.

Loraine. Your observations were made mainly in normal subjects. Do you have any information on blood levels in pathological conditions?

Tait No.

Heller. Dr. Tait, have you had occasion to look for diurnal changes in your normal subjects?

Tait. Yes, in normal subjects with uncontrolled diet, such as those whose daily rate of production of aldosterone was measured, and in normals on a controlled diet and fully reclining, consecutive 8-hour collections showed little variation, particularly compared with that of free cortisol. The small variation that did occur was not correlated with cortisol, sodium or potassium. Venning and Luetscher also found this.

Pearlman: It might be interesting to compare for the sake of completeness the turnover time of endogenous aldosterone in the blood pool with that of the other steroid hormones. The figure previously given for progesterone and the oestrogens is 3 to 6 minutes, that for cortisol is 26–47 minutes, and on the basis of Dr. Tait's figures we would arrive at a turnover time for aldosterone of about 6–10 minutes. In other words, the turnover time of aldosterone is much closer to that of the rapidly disappearing steroid hormones such as progesterone and the oestrogen, rather than that of cortisol.

Morris: In the days when Dr. Williams and I were working in this field, there was some conflict between Bush and ourselves over the different levels of corticosterone in plasma. I think that I must accept Dr. Tait's elegant work as being quite definitive here, and that we were wrong and Bush was certainly right. At the time I thought the evidence was fairly well balanced.

Bush: I cannot let Prof. Morris's handsome withdrawal go by without thanking him (a) for laying a long-lived bogey in my mind, and (b) pointing out that his original criticism of our method at the time was quite justified. It was only when Sandberg and I repeated the work with more adequate controls that we really became happy that probably our figures were all correct. I think it does justify my feelings expressed the other day, that it is nice when we can resolve these conflicts in assay, as it saves a lot of other people a lot of trouble, and it leaves them free to do other more useful things.

Short Communication:

THE DETERMINATION OF PLASMA OESTROGEN LEVELS IN LATE PREGNANCY*

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Medical Unit, London Hospital

WE would like to present our preliminary results in six cases in

The technique used was as follows:

About 9 ml. late pregnancy plasma was refluxed for 45 minutes in 1·8N-HCl. It was hoped that this procedure would result in the release of all oestrogen bound to protein, in addition to the hydrolysis of conjugates. Pure oestrogens appear to be stable to this treatment

The resulting extract was subjected to partition chromatography as follows. The extract was transferred to a partition column, 14 cm. long, 0·57 cm. internal diameter, containing "Hyflo Supercel" as supporting phase, intimately mixed with the stationary phase—72 per cent methanol: 28 per cent distilled water (v/v). Chromatography was carried out using three successive mobile phases, of the following compositions:

First mobile phase—20 per cent CCl_4–80 per cent petroleum ether (b.p., 60°–80°).
Second mobile phase—14·6 per cent CCl_4–13 per cent $CHCl_3$–72·4 per cent petroleum ether.
Third mobile phase—48 per cent $CHCl_3$–52 per cent petroleum ether.
(All percentages are v/v).

The composition of the second mobile phase was obtained by mixing the first and third mobile phases, in the proportions 73 per cent and 27 per cent respectively. The successive mobile phases were

* This short communication was presented at the Conference by Prof C. J. O. R. Morris.—ED.

delivered to the column by automatic means. A brief description of this chromatographic procedure is given by Aitken and Preedy (1956), using stationary and mobile phases of slightly different composition.

One ml. fractions obtained from the column were dried off and the
d fluorimetrically by the method
except that benzene was substi-
thanol mixture used to dissolve
the dry residues before fluorimetry.

The degree of separation of the three oestrogens, oestrone, oestradiol-17β and oestriol, using the above procedure, is shown in Fig. 1. Quite small amounts of oestrogen, 0·1 μg. or less, can be

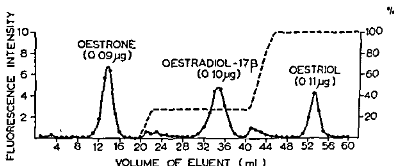


FIG 1. Separation of the three main oestrogens by partition chromatography. The oestrogen peaks are labelled The interrupted line indicates changes in polarity of the mobile phase (see text).

conveniently estimated. The interrupted line indicates the introduction of the third and more polar mobile phase into the system,
increase in
The small
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he column
when the polarity changes.

Fig. 2 (upper curve) shows the result of our procedure in late
[illegible] plasma using 9 ml. The three oestrogen peaks are labelled

interfering material.

A further *9 ml. of the same plasma was submitted to the same* procedure, but before the extract was chromatographed approximately 0·24 μg. oestrone, 0·12 μg. oestradiol-17β and 0·38 μg.

oestriol were added. The result is shown in Fig. 2 (lower curve). It will be seen that the addition of the pure oestrogens results in a symmetrical increase in size of the three labelled peaks.

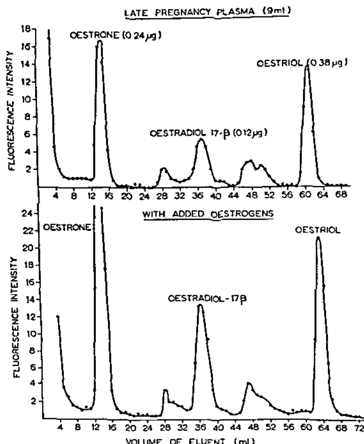


FIG 2. Upper curve. A typical result obtained from the chromatography of late pregnancy plasma.

Lower curve. The addition of crystalline oestrone, oestradiol-17β and oestriol to the same plasma results in a symmetrical increase in size of the labelled oestrogen peaks.

The plasma levels of oestrone, oestradiol-17β and oestriol in the six cases of late pregnancy which we have studied are given in

17

six normal males have also been studied. In some no oestrogen could be detected, but in others oestrone and oestriol of the order of 0·1–0·2 μg /100 ml. plasma was found. No oestradiol-17β could be

for urine, that is a mean of 82 per cent (Aitken and Preedy, 1956).

Table I

PLASMA-OESTROGEN LEVELS IN NORMAL LATE PREGNANCY
(Reproduced by permission of the Editor, *The Lancet*)

Case No	*Age*	*Week of pregnancy*	*Primiparous (P) or multiparous (M)*	*Oestrogen levels (μg per 100 ml. plasma)*		
				Oestrone	*Oestradiol-17β*	*Oestriol*
1	29	41	M	2 65	1·25	4 28
2	25	38	P	3 06	2·04	17 5
3	34	38	M	7 15	2·19	7 10
4	36	38	M	10 30	1·40	8·65
5	22	39	P	9 20	2·93	11·7
6	36	42	M	6·50	1·82	8·65

It appears likely that this procedure will detect one of the newly

urine.

REFERENCES

62, 15P.
ANSON, I. T.
OISY, E. A.
91.

GENERAL DISCUSSION

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Table I (Loraine)

CONCENTRATION OF OESTROGENS IN PREGNANCY BLOOD

Source	*Oestrogen concentration µg./100 ml*		
	Oestriol	*Oestrone*	*Oestradiol–17β*
Cord blood	38–70	1 02–1 8	0 51–0·85
Maternal blood (35–40 weeks' gestation)	5 2–8·0	1·5 –4 6	0·7 –1 4

Table II (Loraine)

RECOVERY OF OESTROGENS FROM BLOOD

Material tested	*Recovery per cent*		
	Oestriol	*Oestrone*	*Oestradiol–17β*
Cord blood	72–108 (100)	65–90 (79)	65–95 (86)
Male blood	51–111 (74)	40–76 (66)	62–82 (71)

Table III (Loraine)

DISTRIBUTION OF OESTROGENS IN WHOLE BLOOD

	Distribution per cent		
	Free	*Conjugated*	*Protein-bound*
Oestriol	21	39	40
Oestrone	80	nil	20
Oestradiol-17β	80	nil	20

Diczfalusy: It was very gratifying to see Dr. Preedy's nice data, but I should like to point out one discrepancy. My analyses in 1953 (*Acta endocr.*, **12**, suppl. XII,) were carried out on so-called retroplacental blood, i.e. on vaginal blood following delivery; therefore one would expect somewhat higher values here in the immediate neighbourhood of the placenta than in the systemic circulation. Last year we reported an improved method for the estimation of oestrone, oestradiol-17β and oestriol in tissues (Diczfalusy and Lindkvist (1956). *Acta endocr.*, **22**, 203). Recently this method has been applied to ... mogens ... In very ... n and I fo ... blood, "f ... tween 3· ... to 4·0 µg ... l-17β, ... er in ... Dr. ... Bush pump pumps it in. I noticed, Dr. Bush, that you quoted a value of ... der ... his ... is means ... being carried in the blood, five-sixths of it is carried in the red cell? Or would you care to go that far?

Bush: No, I wouldn't. I think there are two things: first, *in vivo* you have a steady state in which the concentration of *freely extract-*

able cortisol from red cells would appear to be very low; but in view of the low recoveries of cortisol I have described from lysed cells, I think those results by direct analysis of red cells may need further examination. Certainly I regard my own figures on this question as subject to considerable doubt and intend to repeat the work on larger volumes of cells, correcting with the recovery for the radioactive steroid. Second, however, the *in vitro* uptake to my mind

concentration in or on the cells agree with the results calculated from the outside figures, which are not to my mind in doubt. The two possible explanations to my mind are, either that the phenomenon does not exist *in vivo* because the cells have been damaged *in vitro*; or that there may be a metabolic removal of cortisol *in vivo* so that the steady state concentration is low. I think until one has analysed fresh cells with a control on the recovery from these cells, one cannot be sure of the actual content of red cells *in vivo*.

Farrell: Along the same line of questioning, I have noted that in citrated plasma the ratio inside : outside is 0·3 Now that certainly

pending medium is very much greater.

METABOLISM AND PLACENTAL TRANSMISSION OF CORTISOL DURING PREGNANCY, NEAR TERM

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KNOWLEDGE of adrenal physiology during pregnancy and during neonatal life has advanced rapidly in recent years. Such progress was due primarily to the development of methods for the determination of circulating steroid hormones and to improvements in the techniques of measuring their urinary metabolites.

A progressive rise in the levels of free plasma 17-hydroxycorticosteroids (17-OH-CS) during the course of pregnancy has been observed by several investigators. Gemzell (1953) reported a fourfold increase in plasma 17-OH-CS near term when compared to the levels observed early in pregnancy. At 30–40 weeks of gestation and prior to the onset of labour, Assali, Garst and Voskian (1955) found an average level of 49 μg. per 100 ml. plasma and Robinson and collaborators (1955) an average of 33 μg. Bayliss and co-workers (1955) observed an increase from control values of 10–13 μg. to an average of 24 μg. per 100 ml. In our experience, the 8 a.m. levels of free plasma 17-OH-CS, near term, were somewhat less elevated than those reported by most investigators cited above. At 37–40 weeks of pregnancy, the average in 28 subjects was 23·3 μg. with a standard deviation of ± 9·2, compared with an average of 15·5 ± 6·3 μg. per 100 ml. plasma in a group of 54 non-pregnant females. This discrepancy in results must be related to differences in techniques even though most of the other workers were using the method of Nelson and Samuels (1952) or a modification thereof. In

the present study, the modification of Eik-Nes, Nelson and Samuels (1953) was employed; a satisfactory correlation existing between results obtained by this method and paper chromatographic techniques in normal individuals before and after ACTH administration (Bush and Sandberg, 1953), as well as in patients undergoing surgical and medical stress (Migeon *et al.*, 1956*b*). As shown in Table I, in two women

Table I

COMPARISON OF THE RESULTS OBTAINED BY THE METHOD OF NELSON AND SAMUELS AND PAPER CHROMATOGRAPHIC TECHNIQUES†

*Sample**	*Delivery*	*17-OH-CS µg./100 ml. (Nelson and Samuels)*	*Compound F µg./100 ml. (paper chromatography)*
Maternal plasma	Elective Caesarean	23·5	18 0
Maternal plasma	Elective Caesarean	26 0	19 8
Maternal plasma	Vaginal delivery	56 0	47·6
Cord plasma	Vaginal delivery	21 0	14 5

* Each sample was made up by pooling approximately equal amounts of plasma from three mothers or three umbilical cords.
† Technique described by Migeon and co-workers (1956*b*)

near term (prior to an elective, repeat Caesarean section) the values obtained for 17-OH-CS were in good agreement with the amounts of cortisol eluted from the paper chromatograms of corresponding extracts. It is therefore our feeling that, at the end of pregnancy, the levels of free plasma 17-OH-CS are not more than twice the control values.

It has been observed that labour also causes a rise in the maternal concentrations of plasma 17-OH-CS. This is illustrated in Table II by the higher pre-delivery values in the vaginal series when compared to those obtained in the elective Caesarean group. Our findings confirmed those of other investigators (Assali, Garst and Voskian, 1955; Gemzell, Robbe and Ström, 1956).

The trauma of parturition in itself appeared further to increase the maternal levels as shown by the higher concentrations

found at delivery when compared to the pre-delivery values in the vaginal series (see Table II), although this might be only the culminant effect of labour.

Following delivery, the plasma 17-OH-CS levels were reported to remain elevated for a period of time (Robinson *et al.*, 1955). In our experience (Migeon *et al*, 1956*a*), on the third day post-delivery, the maternal concentrations were found to be higher than normal in the vaginal series (Table II).

From these data, it has been assumed that there was a progressive increase of the rate of corticosteroid production during the course of pregnancy. Other evidence was given by the study of urinary excretion of corticoids by pregnant women: glucocorticoid excretion has been reported to be increased by Venning (1946); urinary neutral reducing lipids were also found to be elevated (Heard, Sobel and Venning, 1946; Jailer, 1951; Devis, 1954). An elevation in the urinary levels of formaldehydogenic substances and the steroids reacting with dinitrophenylhydrazine has also been reported by Tobian (1949), and by Jayle and co-workers (1953), respectively.

Several theories have been presented to explain these results. An increase in size of the maternal adrenals has been mentioned by various authors (see review by Whiteley and Stoner, 1957), and has been taken as evidence of hyperfunction during pregnancy; this would be the result of the high levels of oestrogens in maternal and foetal blood (Gemzell, 1953).

It has also been postulated that the placenta was the site of corticosteroid biosynthesis and that, consequently, the elevated plasma 17-OH-CS observed during gestation might be the result of an extra-adrenal source of hormones. Such a theory is sustained by the fact that adrenal cortical hormones were detected in human placenta extracts by Johnson and Haines (1952) and by de Courcy, Gray and Lunnon (1952). Furthermore, the findings of Knowlton, Mudge and Jailer (1949), those of Jailer and Knowlton (1950), Hills and his associates (1954) and Jailer (1956) suggested that in pregnant Addisonian women there was some evidence of adrenocortical-like activity, presumably derived from the placenta.

Table II

PLASMA 17-HYDROXYCORTICOSTEROID LEVELS IN CASES OF VAGINAL DELIVERIES AND CAESAREAN SECTIONS

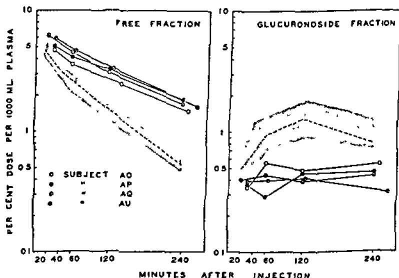
Delivery	*Case Number*	*Age (yrs)*	*Race*	*Duration of Pregnancy (Weeks)*	17-OH-CS (μg. per 100 ml. plasma)				
					Maternal		*Cord*	*Maternal Post-Delivery*	
					Pre-Delivery	*At Delivery*		*3rd Day*	*7th Day*
Vaginal	31 D B	37	C	39	26·4	38·5	10·9	23·2	
	32 E B.	36	C	37	24·0	41·5	18·6	14·9	
	33 P M.	15	C	36	53·0	46·0	0·2	21·5	
	34 M W	22	C	39	47·5	52·0	27·0	43·0	
	35 F S	32	C	41	19·5	54·4	12·0	5·9	
	36 O.D	35	C	41	41·8	56·0	16·5	16·5	
	37 M M	27	C	32	52·5	67·0	43·5	40·0	
	38 M.R	28	W	37	71·0	76·1	63·8	30·5	
	39 J R.	22	W	39	69·0	78·0	14·3	29·4	
Indicated	40 S M	32	W	42	23·0	28·0	5·0	—	29·2
	41 S.W	29	C	40	19·0	34·0	7·0	14·0	15·8
	42 H.M	22	W	35	31·0	47·0	22·0	53·2	29·7
Caesarean	43 M P.	32	W	43	—	66·0	16·5	6·4	14·0
	44 I G.	30	W	44	48·0	98·5	13·6	18·0	16·5
Elective	45 E.McL	27	C	19	6·8	8·6	0·6	—	—
	46 G J.	25	W	38	14·0	20·6	4·3	25·0	20·3
	47 C L.	34	W	38	13·6	21·5	1·4	23·5	10·5
Repeat	48 P G	26	W	39	17·0	23·0	5·0	16·0	13·2
	49 E J	20	C	38	10·2	32·4	9·5	—	—
Caesarean	50 J.B.	26	W	39	18·4	33·5	8·0	18·4	19·4
	51 D G.	28	C	38	24·4	35·8	0·3	—	—

The ovaries have also been proposed as a possible site of the increased corticoid activity during gestation (Devis, 1949).

As a result of this information, we undertook the study of the metabolism of [4-^{14}C]cortisol in pregnant women.

Study of the metabolism of [4-^{14}C]cortisol during pregnancy, near term

One μc of the radioactive steroid was administered 24–48



hours prior to "elective, repeat Caesarean section" (Migeon, Bertrand and Wall, 1957).

The disappearance curves of free radioactivity in the plasma and those of the *glucuronoside fractions are shown* in Fig. 1. The results are expressed in percentage of the dose administered per 1000 ml. plasma and are plotted against time. The shaded areas show the extreme range of variation obtained

in five normal males and two non-pregnant females (Migeon *et al.*, 1956c), while the broken lines show the average values for the control group. The half-life of the free fraction is 80 minutes for the controls in contrast to 140 to 160 minutes in the four mothers. This means that a pregnant woman near term who has a cortisol production equal to that of a non-pregnant individual, could have a level of free plasma 17-OH-CS twice that found in the control subject. It follows

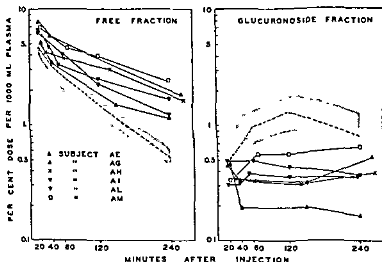


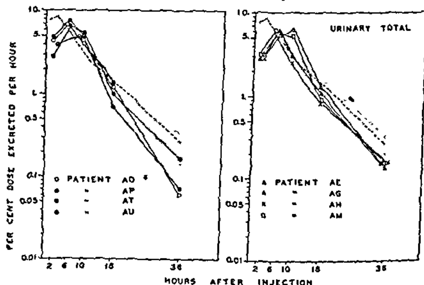
FIG. 2. Radioactivity in the free and glucuronoside fractions from plasma of mothers who received [4-^{14}C]cortisol 17 to 69 minutes prior to an elective repeat Caesarean section

that this alteration of cortisol metabolism could be entirely responsible for the magnitude of the increase in plasma 17-OH-CS observed at the end of pregnancy by Bayliss and collaborators (1955) and by ourselves. However, it would not completely account for the three- to fourfold increase reported by Gemzell (1953) and Assali, Garst and Voskian (1955). It is pertinent here, of course, that the glucuronoside fraction was much smaller than normal.

In Fig. 2 are given the curves obtained in six cases where

the radioactive steroid was given 17–69 minutes prior to elective, repeat Caesarean section. The results were similar to those found in patients who had received [4-^{14}C]cortisol 24–48 hours prior to delivery; the rise in plasma 17-OH-CS levels observed following this surgical procedure would therefore appear to be due to a true increase in adrenal function.

We also studied the urine in these subjects. Collections were



patients AE, AG, AH, and AM [illegible]

made 4, 8, 12, 24 and 48 hours after the administration of [4-^{14}C]cortisol. The total radioactivity of each sample, as well as the radioactivity of the free fraction, glucuronoside fraction, continuous ether extraction at pH 0·8 and the strong acid hydrolysis fractions were determined. The technique of preparation of the various extracts has been described elsewhere (Migeon *et al.*, 1956*c*).

In Figs. 3 to 6, the rates of excretion of radioactivity per hour are plotted against time. The shaded area shows the

range of variation obtained in seven normal individuals; the broken line representing the average. The full lines are the curves obtained in the pregnant subjects. On the left are the cases given isotopic cortisol 24 to 48 hours prior to Caesarean section, and on the right, those to whom it was administered 17 to 69 minutes prior to section.

During the first 4 hours following [4-^{14}C]cortisol injection,

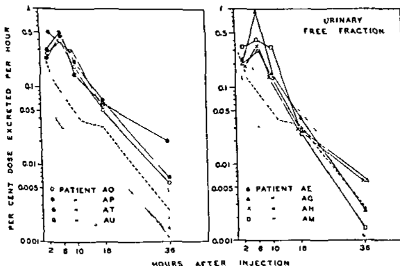


FIG 4 Rate of urinary excretion of free radioactivity (see comments for Fig 3).

the rate of excretion of total radioactivity (Fig. 3) was one-half of that found in normal subjects. After this initial period, the rates observed in pregnant women near term were not significantly different from those found in the control group. The low excretion of radioactivity during the first 4 hours accounts for the fact that the total 48-hour urinary excretion was only 64 to 78 per cent of the dose compared to an average of 88 per cent for the controls.

There was some decrease in the excretion rate of the glucuronoside fraction during the first 8 hours, and after this period of time the rate was at the lower limits of the normal range (Fig. 5). These findings correlate with the levels obtained from plasma.

The amounts of radioactivity obtained after 48-hour continuous ether extraction at pH 0·8 (Fig. 6) were somewhat

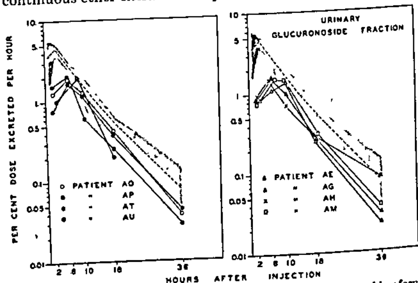


FIG.

larger than normal during the 4 to 8 hour collection period; thereafter, the rate of excretion was variable.

If alteration in the metabolism of cortisol was the main cause of the increase in plasma 17-OH-CS concentrations and if, consequently, cortisol production was not significantly changed during pregnancy, it would follow from our data on urinary excretion of radioactivity that the urinary excretion of corticosteroids in pregnant women should not be elevated. This is in contradiction to the results reported in the literature. However, a critical examination of these results can explain the apparent difference in findings. In the bioassay

employed by Venning the free corticosteroids are not removed prior to pH 1·0 hydrolysis, and it is probable that cortisol and cortisone are biologically more potent than their tetrahydro derivatives (Venning, Kaznin and Bell, 1946). It is conceivable, therefore, that the increase in glucocorticoid concentration observed by this author might be related to the increased proportion of free compounds excreted during the terminal

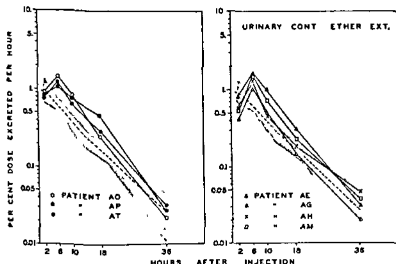


FIG. 6 Rate of urinary excretion of radioactivity following 48-hour continuous ether extraction at pH 0·8 (see comments for Fig. 3)

phase of pregnancy. Methods which measure neutral reducing lipids and formaldehydogenic steroids are known to be relatively non-specific. It is possible that some of the other steroids and/or their metabolites which are produced and excreted in large amounts during pregnancy might interfere with the measurement of urinary corticosteroids by these techniques. On the other hand, the determination of urinary 17 : 21-dihydroxy-20-ketones by the reaction of Porter and Silber (1950) is probably somewhat more specific, even though it does not measure all the metabolites of cortisol, such as cortol or cortolone which have been recently isolated (Fukushima

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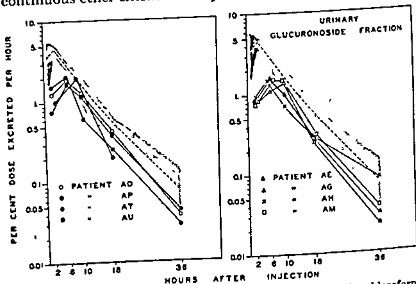


FIG. 5 Rate of urinary excretion of radioactivity extractable by chloroform following β-glucuronidase hydrolysis (see comments for Fig. 3).

larger than normal during the 4 to 8 hour collection period; thereafter, the rate of excretion was variable.

If alteration in the metabolism of cortisol was the main cause of the increase in plasma 17-OH-CS concentrations and if, consequently, cortisol production was not significantly changed during pregnancy, it would follow from our data on urinary excretion of radioactivity that the urinary excretion of corticosteroids in pregnant women should not be elevated. This is in contradiction to the results reported in the literature. However, a critical examination of these results can explain the apparent difference in findings. In the bioassay

Since it is very improbable that the ovary produces any corticosteroids, it appears that, during pregnancy near term, the maternal adrenals are the main site of 17-OH-CS production, but this production is not increased

Transplacental passage of cortisol near term

Another aspect of our work was the study of the transplacental passage of corticosteroids.

Our attention was first attracted by the relationship existing

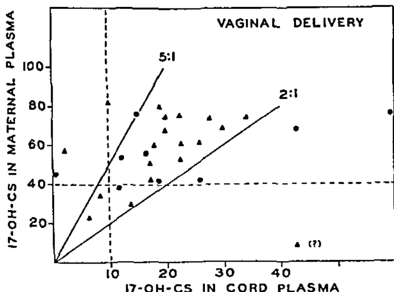


Fig. 7

between maternal and cord levels of plasma 17-OH-CS. As shown in Fig. 7, when the maternal levels were over 40 µg. per 100 ml plasma in cases of vaginal delivery, the cord blood levels were over 10 µg. On the other hand, in cases of elective,

et al., 1955). When using the latter technique, little or no increase in urinary corticoids was observed by Jayle and coworkers (1953) and by Devis (1954). Furthermore, Gray (1954) found that free cortisone excretions were higher than normal while the concentration of tetrahydrocortisone, obtained only after hydrolysis, was in the control range or perhaps slightly lower. Our data entirely confirm these findings.

In view of our results, one can also discuss the various theories of the origin of corticosteroids during pregnancy.

Recently, Whiteley and Stoner (1957) have investigated the effect of pregnancy on human adrenal cortex—they observed a slight increase in weight of the adrenals, but the difference with that of non-pregnant females was not statistically significant. Furthermore Vogt (1957) has shown that oestrogen therapy decreases rather than increases corticosteroid output by the adrenals. Therefore it seems that the maternal adrenals during pregnancy are not in a state of hyperfunction.

The human placenta contains 60–100 ml. maternal blood and the small amounts of corticosteroids isolated from this organ might come from the blood contained in placental tissue.

The increase in corticosteroid excretion by pregnant Addisonian women reported by various authors might also be related to the fact that the techniques of analysis used by these workers were measuring steroids other than cortisol or its metabolites. Beaulieu, Bricaire and Jayle (1956) failed to detect any urinary 17-OH-CS in a pregnant Addisonian when cortisone therapy was withdrawn; Kaiser (1956) did not observe any increase in the levels of urinary 17-OH-CS of a patient maintained on a constant dose of cortisone. The progressive rise in plasma 17-OH-CS found by Jailer (1956) in a similar case with constant steroid therapy can be explained by our findings of a longer cortisol half-life during pregnancy near term. It has been shown that pregnancy does not protect the Addisonian mother against the development of acute adrenal cortical insufficiency (Hills *et al.*, 1954; Kaiser, 1956). Therefore, it seems that the placenta produces little or no corticosteroids.

case, the response of the mothers was similar to that of the non-pregnant subjects. On the right in Fig. 9 are the 17-OH-CS levels in cord blood samples obtained after ACTH infusion to the mother, compared to the cord concentrations obtained when the mother had not received ACTH. It is apparent that ACTH administration to the mother produced

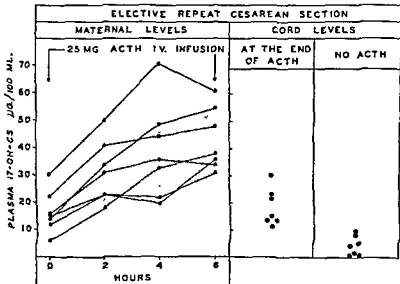


FIG. 9. Effect of ACTH infusion to mothers prior to an elective Caesarean section on maternal and cord levels of plasma 17-hydroxycorticosteroids. The end of the ACTH infusion corresponded to the time of delivery. The cord concentrations were more than 10 µg. per 100 ml. plasma.

an increase in the 17-OH-CS levels in the cord. This effect could result from the transplacental passage of ACTH from the mother to the foetus, and activation of the foetal adrenals. However, it is more likely that it was due to an increased passage of corticosteroids from the mother to the baby.

Cortisol was then administered—50 and 100 mg.—to mothers 30 minutes prior to elective, repeat Caesarean section. As expected, the maternal 17-OH-CS levels were very high (Table III). Of interest here is the fact that cord levels

repeat Caesarean section (Fig. 8), most of the maternal concentrations were under 40 μg. per 100 ml. plasma while those in cord blood were less than 10 μg. In most cases the cord blood values were one-half to one-fifth of those of the mother.

Attempts were then made to increase the 17-OH-CS

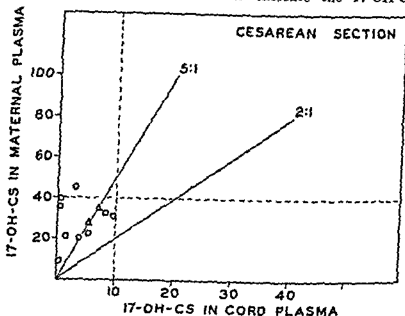


FIG. 8. Relationship between the ... in maternal plasm ... Caesarean section, ... 40 μg. per 100 ml

concentrations of the mother by either ACTH or cortisol administration in order to see if a parallel change would take place in cord blood.

The shaded area in Fig. 9 shows the value of one standard deviation above and below the mean 17-OH-CS response in a large group of controls. It may be noted that, except for one

were higher than any of the values obtained not only in cases of elective section but also in cases of vaginal delivery. Furthermore, these levels were, in all cases, one-half to one-fifth those found in the mother.

In order to prove beyond any reasonable doubt that cortisol can cross the placental barrier, cord blood samples were collected following [4-^{14}C]cortisol administration to the mother. In Table IV is given the free radioactivity in maternal and

Table IV

RELATIONSHIP BETWEEN FREE RADIOACTIVITY IN MATERNAL PLASMA AND THAT FOUND IN CORD PLASMA FOLLOWING ADMINISTRATION OF [4-^{14}C]CORTISOL TO THE MOTHER PRIOR TO AN ELECTIVE REPEAT CAESAREAN SECTION

Subjects	*Time between Injection and delivery*	*% Dose per 1000 ml plasma*		*Ratio Mother: Cord*
		Mother	*Cord*	
A. G.	17 min.	7 70	1 61	4·78
A. H.	27 min.	4·19	1 46	2 87
A E.	39 min	3 46	1 21	2·86
A. L	51 min.	3 29	1 57	2·09
A. I.	60 min.	4·11	0 80	5 14
A M.	69 min	4 64	1 70	2 73
A. O	24 hr	0	0·	—
A. Q	46 hr.	0	0·	—
A. P	48 hr	0·	0·	—
A T.	48 hr.	0·	0	—
A. U.	44 hr.	0	0	—

cord f the dose per l.
plasm to reach the cord
very gnificant amounts
17 minutes after the injection to the mother. The ratio of maternal to cord values of free plasma radioactivity was in the

Table III

PLASMA 17-HYDROXYCORTICOSTEROID LEVELS IN MATERNAL AND CORD PLASMA FOLLOWING CORTISOL ADMINISTRATION 30 MINUTES PRIOR TO AN ELECTIVE REPEAT CAESAREAN SECTION

Case Number	*Age (Yrs)*	*Race*	*Duration of Pregnancy (Weeks)*	*Medication*	*17-OH-CS µg per 100 ml. plasma*		
					Maternal		*Cord*
					Control	*On Cpd F*	
59 M.S.	39	W	39	50 mg i.v cpd. F	21 1	178 0	50 4
60 M P.	31	W	38	50 mg. i.v cpd. F	29 2	158·0	67·0
61 A A.	20	W	39	50 mg i v. cpd F	17 6	148 0	77·0
62 S C.	23	C	39	100 mg i v. cpd. F	25 4	301 0	86 5
63 A.J.	36	C	38	100 mg i v cpd F	24 0	326 0	105 0

(c) Finally, the fact that no free or glucuronoside radioactivity was found in the foetal circulation 24 to 48 hours after injection suggests that the foetus has means of disposing of cortisol and its metabolites. They could either be excreted by the foetus or returned to the mother.

Conclusions

1. Pregnancy, near term, does not appear to be accompanied by an increased corticosteroid production. The elevated levels of plasma 17-OH-CS observed in this condition seem to be mainly related to a disturbance in cortisol catabolism characterized by a slower rate of reduction and/or conjugation of this steroid.

2. Cortisol can cross the "placental barrier", but in a definite maternal : cord plasma ratio. Consequently, the levels of 17-OH-CS in cord plasma would seem to be merely the reflection of maternal concentrations

Acknowledgements

The authors are indebted to Dr. Lawson Wilkins and Dr. Nicholson

REFERENCES

ASSALI, N S, GARST, J. B, and VOSKIAN, J. (1955). *J. Lab clin Med.*, 46, 385.

BAYLISS, R I. S, BROWNE, J. C McC, ROUND, B. P, and STEINBECK, A W. (1955). *Lancet*, 1, 62

BEAULIEU, E. E., BRICAIRE, H, and JAYLE, M. F. (1956). *J. clin. Endocrin. Metab.*, 16, 690.

BUSH, I E., and SANDBERG, A A. (1953) *J. biol. Chem*, 205, 783.

DE COURCY, C., GRAY, C. H, and LUNNON, J. B (1952). *Nature, Lond*, 170, 494.

DEVIS, R (1949). *Ann. Endocr., Paris*, 9, 173

DEVIS, R. (1954). *Gynéc et Obstét*, 53, 57.

EIK-NES, K, NELSON, D H, and SAMUELS, L T. (1953). *J. clin. Endocrin Metab*, 13, 1280.

administered 24 to 48 hours prior to delivery, no radioactivity was detectable in either maternal or cord blood.

In a paper chromatographic study of maternal and cord extracts, 57 and 42·5 per cent of the free radioactivity, respectively, moved in the area corresponding to cortisol.

In Table V are given values representing the plasma

Table V

RELATIONSHIP BETWEEN GLUCURONOSIDE RADIOACTIVITY IN MATERNAL PLASMA AND THAT FOUND IN CORD PLASMA FOLLOWING ADMINISTRATION OF [4-14C]CORTISOL TO THE MOTHER PRIOR TO AN ELECTIVE REPEAT CAESAREAN SECTION

Subjects	*Time between injection and delivery*	*% Dose per 100 ml plasma*		*Ratio Mother, Cord*
		Mother	*Cord*	
A. G.	17 min	0 46	0·13	3 54
A. H	27 min.	0 34	0 09	3·78
A. E	39 min.	0 19	0 09	2·11
A L.	51 min.	0 38	0 08	4 75
A I.	60 min.	0 48	0·13	2 92
A M	69 min.	0 56	0 24	2 33
A O.	24 hr.	0	0·	—
A Q.	46 hr	0	0·	—
A P.	48 hr	0	0·	—
A T.	48 hr.	0	0	—
A U.	44 hr.	0	0	—

glucuronoside fractions. These levels are low, but a significant amount of radioactivity was found in the cord blood, while 24 to 48 hours after injection of [4-14C]cortisol, no radioactive glucuronosides were detected.

From the data presented it may be concluded that:

(*a*) [4-14C]cortisol can cross the "placental barrier".

(*b*) The fact that radio-conjugates were also detected in the cord blood indicates either that they can also cross the placental barrier or that the foetus has some means of metabolizing the steroid.

amniotic fluid in toxaemia and in non-toxaemic women. Do your observations offer any clarification here?

Migeon: We have tried, on two occasions, to measure the 17-hydroxycorticosteroids of amniotic fluid by the method of Glenn and Nelson (1953. *J. clin. Endocrin. Metab.*, 13, 911) but have found only negligible amounts.

When we gave [4-^{14}C]cortisol to the mother, 17 to 69 minutes prior ... When the ... cted a very ... se adminis-

Diczfalusy: Hoet's work was published in *Experientia* (Hoet and Osinski (1954) *Experientia*, 10, 467). It involved chromatographic

adrenal function of the foetus.

First, in cases of "elective, repeat, Caesarean section" (that is to say when no labour has taken place and when there was little maternal stress), the 17-hydroxycorticosteroid concentrations in cord plasma were

normal adults

FUKUSHIMA, D. K., LEEDS, N. S., BRADLOW, H. L., KRITCHEVSKY, T. H., STOKEM, M. B., and GALLAGHER, T. F. (1955) *J. biol. Chem.*, **212**, 449.
GEMZELL, C. A. (1953) *J. clin. Endocrin. Metab.*, **13**, 898.
GEMZELL, C. A., ROBBE, H., and STRÖM, G. (1956). *Acta Endocr., Copenhagen*, **23**, 158.
GRAY, C. H. (1954). *Ann. Endocr., Paris*, **15**, 22.
HEARD, R. D., SOBEL, H., and VENNING, E. H. (1946). *J. biol. Chem.*, **165**, 699.
HILLS, A. G., VENNING, E. H., DOHAN, F. C., WEBSTER, G. D. Jr., and RICHARDSON, E. M. (1954). *J. clin. Invest.*, **33**, 1466.
JAILER, J. W. (1951). Proc. II Clinical ACTH Conference, **1**, 77. New York: Blakiston Co.
JAILER, J. W. (1956). *In* Adrenal function in infants and children, p. 80, ed. Gardner, L. I. New York Grune & Stratton.
JAILER, J. W., and KNOWLTON, A. I. (1950). *J. clin. Invest.*, **29**, 1430.
JAYLE, M. F., DESGREZ, P., SERPICELLI, J., and ROZED, J. (1953). *Ann. endocr., Paris*, **14**, 877.
JOHNSON, R. H., and HAINES, W. J (1952). *Science*, **116**, 456.
KAISER, I. H. (1956). *J. clin. Endocrin. Metab.*, **16**, 1251.
KNOWLTON, A I., MUDGE, G. H., and JAILER, J. W. (1949). *J. clin. Endocrin*, **9**, 514.
MIGEON, C. J, BERTRAND, J., and WALL, P. (1957). *J. clin. Invest.* In press.
MIGEON, C. J., PRYSTOWSKY, H., GRUMBACH, M. M., and BYRON, M. C. (1956*a*) *J clin. Invest.*, **35**, 488.
MIGEON, C. J., and SANDBERG, A. A., BLISS, E. L., and KELLER, A. R. (1956*b*). *J. clin. Endocrin. Metab.*, **16**, 253.
MIGEON, C. J., SANDBERG, A. A., DECKER, H. A., SMITH, D. F., PAUL, A. C., and SAMUELS, L. T. (1956*c*). *J. clin. Endocrin Metab.*, **16**, 1137.
NELSON, D. H., and SAMUELS, L. T. (1952). *J. clin. Endocrin. Metab.*, **12**, 519.
PORTER, C. C., and SILBER, R H. (1950) *J. biol. Chem*, **185**, 201.
ROBINSON, H. J., BERNHARD, W. G., GRUBIN, H., WANNER, H., SCWEKOW, G. W., and SILBER, R. H (1955). *J. clin. Endocrin. Metab.*, **15**, 317
TOBIAN, L. Jr. (1949). *J. clin. Endocrin.*, **9**, 319.
VENNING, E. H. (1946). *Endocrinology*, **39**, 203.
VENNING, E. H., KAZNIN, V E, and BELL, C. J. (1946). *Endocrinology*, **38**, 79.
VOGT, M. (1957). This volume, p. 193.
WHITELEY, H. J., and STONER, H. B. (1957). *J. Endocrin.*, **14**, 325.

DISCUSSION

whereas we could hardly detect any free oestradiol or oestrone in several foetal organs, in the amniotic fluid, or in the urine of newborn babies; so the question arises, what happens to these free placental oestrogens? Either they do not cross the placental barrier, or if they do—and I think they do—a considerable part of them seems

he receives from his mother. However, we have some information on how the baby might handle, after birth, the cortisol that crossed the placental barrier. When [4-^{14}C]cortisol is administered to the mother

not accounted for.

Diczfalusy Have you studied the placenta?

Migeon. Yes, we have some information on the placentas of mothers who received [4-^{14}C]cortisol 48 hours prior to delivery (that is, at a time when no radioactivity could be detected in the maternal plasma). At that time, the placenta contained significant amounts of radio-

Finally
(also
culata
foetal develops. If one is
willing zona fasciculata is the site of corticosteroid
produc... it may be seen that the histology of the neonatal adrenal fits well with our findings.

However, this is only indirect evidence Furthermore, we do not exclude the possibility of changes in foetal adrenal function when the status of the mother is modified.

Harris. Laboratory data indicates that the pituitary-adrenocortical system of mice, rats and rabbits is active before birth. The hypophysectomized (decapitated) foetal rat or rabbit shows adrenal atrophy, and the unilateral adrenalectomized rat foetus is reported to show compensatory hypertrophy in the remaining adrenal cortex. Also in the human the reduction in size of the adrenocortical glands in the anencephalic monster is usually thought to be due to a pituitary deficiency (for references see *Jost, A.* (1953). *Recent Progr Hormone Res*, *8*, *408*). Have you studied the concentration of adrenal steroids in the blood, or umbilical cord blood, of pregnant Addisonian patients on constant maintenance doses of cortisone?

Migeon Unfortunately, we have not had the opportunity of studying pregnant Addisonian mothers. As we mentioned in our paper, Beaulieu and colleagues failed to detect urinary 17-hydroxycorticosteroids in a pregnant Addisonian when cortisone therapy was withdrawn, furthermore, the administration of ACTH during one of these periods of cortisone withdrawal did not result in urinary excretion of 17-hydroxycorticosteroids

Jailer's patient was receiving a constant dose of cortisone but her plasma 17-hydroxycorticosteroids levels rose progressively during the course of pregnancy. We feel that this phenom...
by our finding of a longer cort...

A certain number of Addi...
of 17-hydroxycorticosteroids i...
does not raise these levels, an...
relative adrenal insufficiency
conceivable that their plasma ...costeroid concentrations would increase somewhat during the course of pregnancy without an increase in corticosteroid production and, consequently, their disease might be favourably influenced

Diczfalusy Do you think that the foetal organ is capable of conjugating corticosteroids?

Migeon *Our data do not enable us to an...*
true that we have detected ...
do not know...
moth...
the n...

Dic... ...of oestrogen, we find a high concentr... ...ree oestrogens (e.g. oestradiol-17β), in the placenta,

If we believe that during late pregnancy cortisol production is not increased, there is no doubt that the plasma levels of 17-hydroxycorticosteroids are elevated. It is also possible that the hormone concentration in the various tissues is increased, and that might be why arthritis [illegible]

[illegible]

chemical reaction that is going on in the tissues? Certainly the pregnant woman has not got Cushing's disease, and yet she has a concentration of cortisol in the blood of much the same order as those found in Cushing's disease. It seems to me that it may well be that it is a certain reaction in the metabolism of those steroids which is important in the physiological effect, rather than simply the level of the steroids in the fluids bathing the tissues.

cortisol which you could not account for, either as glucuronide o[r] material. Have you studied any of these blood samples from t[he] blood to know whether there is any considerable amount of radioa[ctivity] unaccounted for by either of these two fractions?

Migeon: No, we have not studied the total radioactivity o[f] plasma.

Samuels. Have you tried strong acid hydrolysis?

Migeon: No, we have not

Samuels: That seems to me to be very important, because either must be different forces causing distribution, or there must be an[other] metabolic route

Prof. Gray, have you studied the excretion of 17-ketosteroids in [an] Addisonian patient? Many years ago, in Minnesota, Dr. Winter a[nd I] followed an Addisonian through pregnancy and found a very defi[nite] rise in 17-ketosteroids during pregnancy, which dropped on deliver[y].

Gray: In that case, no. We have avoided measuring 17-ketostero[ids] in pregnancy because of the interference by other compounds which [are] excreted during pregnancy. You need to go to quite a lot of trouble [to] get rid of these interfering substances before you can measu[re] 17-ketosteroids.

Samuels. That criticism could be made of our work at that time, a[nd] this was before techniques of purification were as good as they ar[e] now.

Bush · The histological changes in the foetal adrenal in sheep are very similar to those in man There is the same large foetal cortex with an eosinophilic zone relatively poor in lipid. Dr. Balfour in Cambridge says that results on adrenal venous blood vary throughout pregnancy and the results for the very end of gestation are rather doubtful, at the moment. Certainly in what would correspond to about the end of the second trimester and beginning of the third trimester in the sheep foetus I could find no detectable cortisol, no detectable Δ_{4} $_{3}$-ketones, and no detectable 17-ketosteroids in adrenal venous blood Dr. Balfour has got many more results than I have, pointing to the same thing So in this phase, in the foetal adrenal of one species, we know very definitely that the secretion rate of steroids one would expect the adrenal to produce is extremely small.

Migeon · The observations are very interesting.

Tait: Dr. Migeon, have you studied an arthritic pregnant woman in this way?

Migeon · No.

Tait: Do you think that thes[e] ... the concent[...]

I[...]
level, it
50 mg ·
producti[...] [re]placing the endogenous

blood, and their results indicated that in pregnant women the circulating levels were very low. Edgar (1953*a*) developed their technique and applied it to the problem of progesterone determination in the blood of domestic animals. He was able to detect the hormone in the blood taken from the ovarian veins of ewes, but he could not find any in the peripheral blood of the pregnant and non-pregnant ewe, mare, cow or sow, using a method with a sensitivity of 0·1 μg./ml. blood (Edgar, 1953*b*). Zander and Simmer (1954) developed a technique with a sensitivity of 0·05 μg. progesterone/ml. blood, and they estimated the progesterone levels in the peripheral blood of women during late pregnancy, the average concentration which they found being 0 142 μg./ml. plasma (i.e. 14·2 μg./100 ml.). However, Zander (1955) was unable to detect progesterone in the blood of non-pregnant women at various stages of the menstrual cycle. Using a combination of Zander's and Edgar's techniques, Raeside and Turner (1955) found progesterone in the blood from the ovarian vein of goats, but they failed to detect the hormone in the peripheral blood of either the cow or the goat.

A number of authors have demonstrated that the human placenta is a rich source of progesterone, and values given by Zander (1956) and Pearlman (1957) indicate that, towards the end of pregnancy in the human, the placenta is releasing as much as 250 mg. progesterone into the maternal circulation every 24 hours. The levels of progesterone in the foetus are even higher than those in the blood coming from the uterine vein, or in the peripheral maternal blood (Zander, 1956), and Forbes (1955) has produced some evidence to indicate that the foetus itself may produce progesterone, in addition to the placenta. Philipp (1936) and Hoffmann (1954) have detected progesterone biologically in the urine of newborn infants, and Hoffmann and Uhde (1956) presented some interesting indirect evidence in favour of progesterone production by the human foetal adrenal. However, Zander (1956) believes that the placenta is the main source of progesterone during pregnancy; as evidence for this view, he cites the fact that the

PROGESTERONE AND RELATED STEROIDS IN THE BLOOD OF DOMESTIC ANIMALS

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Progress in the investigation of the part played by progesterone in reproduction has been hampered for a long time by the lack of a sufficiently sensitive and specific method of assay. The fact that large doses are usually required to produce a biological response has to some extent invalidated the numerous bioassay techniques that are available. However, Hooker and Forbes (1947) developed a microbiological assay capable of detecting 0·0002 μg. of the hormone, and the specificity of the technique seemed high. More recently, Forbes and Zander (1956) have shown that two naturally occurring metabolites of progesterone, the 20-hydroxy derivatives, both give positive responses in the Hooker-Forbes bioassay, the 20β-epimer being twice as active as progesterone in this respect. A number of studies were carried out on the levels of progesterone in the blood of women, sheep and rabbits using the Hooker-Forbes technique, but there was always a marked discrepancy between the results obtained by bioassay and those obtained from chemical determinations. Other biological tests have been applied to the problem of progesterone determination in blood, and Duyvené de Wit (1938, 1941), using the ovipositor response of the bitterling, obtained some slight evidence for the presence of the hormone in the peripheral blood of the cow and the pig; but since oestrogens and androgens gave false positive responses in this test, the validity of his results is questionable.

Butt and colleagues (1951) were the first to develop a reliable chemical method for the assay of progesterone in

the only exception, and even then up to 200 ml. could be handled conveniently. After removal of the ether by evaporating the extract to dryness, the residue was dissolved in 80 ml. light petroleum; the light petroleum was then extracted six times with 10 ml. portions of 70 per cent methanol. In view of the high partition coefficient of progesterone in such a methanol/light petroleum system, over 98 per cent of the progesterone was recovered in the methanolic extracts, together with an even greater percentage of the more polar adrenal steroids. Fat and pigments present in the original ether extract remained in the light petroleum phase, and on evaporation of the methanolic extract and concentration in a small

chrom

systen

α : β-unsaturated ketones were detected on the chromatograms by ultraviolet contact photography; they were then eluted from the chromatogram and measured spectrophotometrically, using a micro-cell of optical depth 1 cm. and capacity 0·5 ml. Using this technique, it was possible to detect less than 1 μg. progesterone; when 5, 10 or 15 μg. progesterone were added to a 250 ml. sample of ox plasma, the recovery rate was of the order of 50 per cent, and there was evidence that the losses occurred mainly during the elution of the chromatogram with absolute ethanol, rather than during the initial ether extraction.

The two main criteria used for the identification of all α : β-unsaturated ketosteroids consisted of the R_F value and the ultraviolet spectrum. However, as it is known that there are at least two other steroids which could be confused with progesterone if these two criteria alone were applied—namely the "unknown" compound reported by Hagopian and coworkers (1956) in human placental perfusates, and Δ''-dehydroprogesterone (pregn-4 : 11diene-3 : 20dione) (Zander and Simmer, 1954), it is desirable to check the identification of the steroids when enough material becomes available by such additional procedures as the infrared spectrum.

excretion of pregnanediol in the maternal urine continues unaltered if the foetus is removed and the placenta left *in situ*, and in addition he has been able to isolate progesterone from a chorion-epithelioma. Although progesterone has been isolated from human foetal fluids (Zander and von Munstermann, 1956), it would appear that the foetus is incapable of converting it to pregnanediol, since pregnanediol has never been detected in the urine of the newborn child (Zander, 1956).

Since the evidence for progesterone production in the placentas of domestic animals was sparse and inconclusive, a study was made of the steroids in the placentas of the mare, cow, sheep, goat, and pig (Short, 1956). Progesterone and 20β-hydroxypregn-4en-3one were readily detected in the mare placenta (Short, 1957), although there was considerably less progesterone than in the human placenta. However, in all the other species mentioned, progesterone was not detected. In view of the fact that the corpora lutea are not essential throughout the whole length of pregnancy in the mare, cow and ewe, it seemed important to establish whether progesterone was in fact the hormone responsible for the maintenance of pregnancy in such animals. This necessitated the determination of blood progesterone levels in these species.

The method described by Zander and Simmer (1954) was first tried, but difficulties were at once encountered when large volumes of plasma were extracted. The volumes of solvent became inconveniently large, and the final extract was so contaminated with fat and pigments that it was completely unsuitable for paper chromatography. A technique was therefore devised which overcame these difficulties. It was found that if approximately 0·45 per cent (w/v) NaOH was added to the plasma, no emulsions were formed on subsequent extraction with an equal volume of ether. During this ether extraction, nearly all the fat and pigments remained in the plasma layer, whilst the steroids passed into the ether. In this manner, volumes of plasma ranging from 14 ml. to 800 ml. could be extracted without any difficulty; human plasma was

the production of progesterone, which passes into the foetal blood and maintains the pregnancy. Presumably, sufficient hormone can diffuse across the placental barrier to exert a direct progestational effect on the endometrium, without a significant amount of the hormone ever appearing in the maternal blood stream. This apparent foetal autonomy raises a number of interesting points concerning the mechanisms underlying parturition or abortion in the mare. As Mayer and Klein (1955) expressed it, "Tout se passe comme si, au moment de l'ovo-implantation, s'installait un mouvement d'horlogerie qui dure un temps donné, celui de la vie propre du placenta."

One is also led to speculate why such high levels of pregnanediol are excreted in the urine of pregnant mares, although no work has ever been done to compare the levels found in pregnant and non-pregnant animals. It is hoped to undertake a study of the pregnanediol content of mare allantoic fluid; if the foetal placenta can produce the 20β-hydroxy metabolite of progesterone, perhaps it can also produce pregnanediol.

The Cow

Adler, De Fremery and Tausk (1934) were the first to demonstrate progestational activity in cow placental extracts, and subsequently Ehrhardt and Hardt (1937) managed to confirm this finding, but only in one out of three placentas that they assayed. McDonald, McNutt and Nichols (1953) demonstrated that the corpus luteum in the cow could be removed after about 200 days' gestation without interrupting the pregnancy in the majority of animals, but they encountered some individual variations.

From these results it would have seemed logical to expect that the cow placenta was capable of producing progesterone during the later stages of pregnancy. It was therefore surprising to find that no progesterone could be detected chromatographically in kilogram quantities of cow placenta obtained late in pregnancy or at parturition. In order to secure more definite information on this point, blood from the uterine vein of a 224-day pregnant cow was assayed for progesterone.

The Mare

In the pregnant mare, the corpus luteum of ovulation lasts for about 35 days, and at the end of this time a second crop of corpora lutea is formed in the ovaries. These secondary corpora lutea subsequently regress, so that at about 200 days of gestation the maternal ovaries consist solely of fibrous tissue, with only a few small follicles present. Ovariectomy may safely be performed at this stage without interfering with the pregnancy (Asdell, 1946). The mare placenta was found to contain progesterone as early as 120 days of gestation, in a concentration of 73 μg./kg. tissue. At 270 days about 250 μg. progesterone/kg. tissue were present, together with a smaller amount of the biologically active progesterone metabolite, 20β-hydroxypregn-4en-3one. It has not been possible, however, to detect any progesterone in 1 l. amniotic or allantoic fluid from a 200-day pregnant mare. The fibrotic maternal ovaries from the same animal contained only about 3 μg. progesterone, whereas mare corpora lutea were found to be a rich source of progesterone, a level of 46 mg./kg. luteal tissue having been found in a normal cyclical corpus luteum.

In view of the occurrence of progesterone in the mare placenta, it was somewhat surprising to find that no progesterone could be detected in the peripheral blood of pregnant mares. The analysis of 410 ml. plasma obtained from the uterine vein blood of a 258-day pregnant mare failed to reveal any progesterone, neither was it possible to detect any of the hormone in 500 ml. plasma samples from the jugular vein and brachial artery of the same animal. However, since the mare placenta is epithelio-chorial in nature, it may be that the placental barrier is comparatively impermeable to the placental steroids; it is certainly known to be impermeable to the maternal γ-globulins. The analysis of hypertrophied foetal gonads has shown them to be devoid of progesterone, in spite of the morphological similarity which exists between them and normal luteal tissue. This could be taken, perhaps, as an indication that, during the late stages of pregnancy in the mare, it is the foetal placental tissue that is responsible for

did not contain a corpus luteum; this has recently been confirmed by Raeside and Turner (1955) in the goat.

Further work is in progress with a view to obtaining a more exact knowledge of progesterone levels throughout the reproductive cycle of the cow, and it is hoped to investigate the hormonal mechanisms for the maintenance of pregnancy in this species in the absence of the corpus luteum.

The Sheep

Ehrhardt and Hardt (1937), studying the progestational activity of sheep placental extracts by bioassay, obtained negative results. But it is known that ovariectomy in the ewe may be performed as early as the fiftieth day of pregnancy without inducing an abortion (Denamur and Martinet, 1955); there is also definite morphological evidence of involution of the corpora lutea by the 126th day of gestation (Grant, 1934).

Using the Hooker-Forbes bioassay technique, Neher and Zarrow (1954) investigated the concentration of "progestin" in the peripheral blood of pregnant ewes. They found (1) that the levels tended to rise during pregnancy, and (2) ovariectomy performed as early as the sixty-sixth day had no effect on the concentration of "progestin" in the blood. They therefore concluded that the placenta must be the major source of "progestin" during the second half of pregnancy in the sheep. Their bioassay results indicated a level of 0·3–2 μg. "progestin"/ml. blood at oestrus, rising to 6 μg./ml. in the luteal phas[illegible]
at 6–[illegible]
rising [illegible]
parturition.

Edgar (1953*b*) in his experiments was unable to detect progesterone in the peripheral blood of pregnant or non-pregnant sheep, using a chromatographic technique which had a sensitivity of 0·1 μg./ml. Moreover, he did not find any progesterone in the blood withdrawn from the uterine vein of a 30-day pregnant ewe. However, he was able to find 2 μg. progesterone/ml. in the ovarian vein blood of a 98-day

However, using the technique described by Zander and Simmer (1954), no progesterone was detected in a 30 ml. *sample of plasma obtained from this* cow. *Two attempts to* demonstrate the presence of progesterone in oestrogen-free extracts of full-term cow placentas, using the rabbit bioassay, met with negative results.

Further experiments were carried out, with the aid of the chemical assay technique described above, in order to determine the levels of progesterone in blood taken from the jugular vein of cattle at various stages of the reproductive cycle.

Table I

The concentration of progesterone in the plasma of cows

Results are expressed in µg. progesterone/100 ml. plasma. Recovery rate 50 per cent.

Cow No.	*Breed*	*Stage of Cycle*	*Vol. of plasma ml.*	*Progesterone µg./100 ml.*
1	Guernsey	72 hr. before ovulation	500	not detectable
2	Guernsey	24 hr before ovulation	500	0 20
3	Guernsey	0·5 hr. after ovulation	500	not detectable
4	Guernsey	12 days after ovulation	621	0 19
5	Guernsey	14 days after ovulation	500	not detectable
6	Guernsey	4 5 months pregnant	500	0·24
7	Guernsey	4·5 months pregnant	500	0·28
8	Ayrshire	6 5 months pregnant	500	0·24
9	Friesian	9 months pregnant	500	not detectable

Although the number of animals investigated is small, it is obvious from the results given in Table I that in certain instances progesterone can be demonstrated in cattle blood. But it appears that there are no marked differences between the levels found in pregnant and non-pregnant animals; this indicates that the placenta was probably not contributing a significant amount of progesterone to the maternal circulation during pregnancy. The presence of progesterone in the blood of an animal the day preceeding ovulation is interesting in the light of Edgar's (1953*b*) findings in the ewe, where he was able to detect progesterone in the blood draining an ovary that

chromatograms. This appeared to be an $\alpha : \beta$-unsaturated ketone because its ultraviolet absorption spectrum had a maximum at 240 mμ. and its R_F value was identical with that of 20α-hydroxypregn-4en-3one(20α-hydroxypregnenone). This latter substance, which is a progesterone metabolite, had previously been reported in human blood and tissues (Forbes and Zander, 1956), as well as in the tissues of eviscerated, adrenalectomized rats treated with progesterone (Wiest, 1956). 20α-Hydroxypregnenone is of considerable interest because it possesses a high degree of biological activity in the Hooker-Forbes test, and its presence in the peripheral blood might help to explain the gap between the high levels of blood "progestin" reported by Neher and Zarrow, and the low levels of blood progesterone found by Edgar. In this connection it is also interesting to note that in the rat at any rate, the $C_{(20)}$ carbonyl group of progesterone can be reduced in the peripheral tissues, thus giving rise to this other biologically active "progestin".

The results obtained from the chemical experiment described above are summarized in Table II. As can be seen

Table II

THE CONCENTRATION OF PROGESTERONE AND 20α-HYDROXYPREGN-4EN-3ONE IN THE PLASMA OF PREGNANT EWES

Results are expressed in μg steroid/100 ml. plasma. Recovery rate 50 per cent

Ewe No.	*Breed*	*Treatment and time of autopsy (days pregnant)*	*Vol of plasma ml.*	*Progesterone μg /100 ml.*	*20α-hydroxy-pregn-4en-3one μg /100 ml.*
1	Welsh Mountain	Ovariectomized Killed day 100	650	0 23	0 31
2	Welsh Mountain	Ovariectomized Killed day 98	570	0 39	0 39
3	Crossbred	Unoperated Killed day 99	500	0 35	0 70
4	Welsh Mountain	Unoperated Killed day 99	500	0 38	0·44
5	Border Leicester	Unoperated Killed day 96	500	0 30	0·38

pregnant animal, which presumably indicated that at this stage the corpus luteum was still actively secreting the hormone. A 3·5 month pregnant ewe also contained appreciable quantities of progesterone in the blood draining the ovaries, one of which contained only Graafian follicles.

Thus there would appear to be a marked discrepancy in the peripheral blood values as established by biological and chemical assays. With reference to this, Neher and Zarrow (1954) point out that there may well be another progestational hormone having an even greater biological activity than progesterone itself.

The present study included a series of experiments designed to test the possibility that progesterone may be produced by the sheep placenta. Several kilogram batches of placental tissue were extracted, and the purified extracts examined chromatographically for the presence of the hormone. In no instance could any progesterone be detected, nor was it possible to obtain a positive bioassay response in a rabbit injected with an oestrogen-free extract of sheep placenta. In order to investigate this problem further, six ewes were mated and 50 days later their peripheral blood was examined for the presence of progesterone. Although the analyses were performed on the plasma from about 700 ml. blood, this amount appeared to be insufficient for the accurate determination of the progesterone concentration. At about the sixtieth day of gestation, the ewes were assigned at random to two experimental groups; one group was ovariectomized, and the other served as a control. In no case was pregnancy affected by the ovariectomy. All six animals were slaughtered at about the hundredth day of gestation, and from each, 500–650 ml. plasma were collected for progesterone assay. Unfortunately, the sample from one animal was lost, but on chromatography, progesterone was detected in each of the other five plasma extracts. This proved (1) that progesterone was actually present in the peripheral blood of sheep, and (2) that it could be produced by an extra-ovarian source. In addition to progesterone, another more polar steroid was detected on all

It is obviously not possible to refute Forbes's claims on such scanty experimental evidence. However, from the ovariectomy results alone it would appear that in the goat there is no significant extra-ovarian source of progesterone.

The Pig

There are no reports in the literature of the effects of ovariectomy on the course of pregnancy in the pig, but Duyvené de Wit (1938), using the bitterling ovipositor test, claims to have detected 20 µg. progesterone in 203 g. pig placental tissue. This he regards as an indication that the organ functions more as a store than as a producer of the hormone. He also claims (1941) to have detected progesterone in the blood of a sow at a level of 40 µg./l.

As a part of the present study, kilogram quantities of pig placentas obtained by Caesarean section were extracted on a number of occasions and examined chromatographically for the presence of progesterone; in no case was it possible to detect the hormone in these extracts. A few assays were carried out on the blood progesterone levels of pigs, and the results

Table III

THE CONCENTRATION OF PROGESTERONE IN THE PLASMA OF SOWS

Results are expressed in µg progesterone/100 ml. plasma. Recovery rate 50 per cent.

Pig No.	*Stage of Cycle*	*Vol. of plasma ml.*	*Progesterone µg /100 ml.*
1	luteal	500	0 83
2	90 days pregnant	500	0·34
3	4 days postpartum	500	not detectable

are summarized in Table III. It is interesting to note that the concentration found in the pig during the luteal phase was higher than that found in the pregnant animal.

from that Table, ovariectomy has little effect on the levels of progesterone or 20α-hydroxypregnenone in the blood. It would therefore appear that the extra-ovarian source of progesterone is able to compensate fully for the removal of the ovaries. The evidence presented here indicates that in the ewe the placenta probably contributes little if any progesterone to the circulation during pregnancy.

The Goat

Ehrhardt and Hardt (1937) obtained inconclusive results from the bioassay of extracts prepared from large quantities of goat placentas, and Meites and co-workers (1951) confirmed previous observations that the corpora lutea are indispensable throughout gestation for the maintenance of pregnancy. Raeside and Turner (1955) detected high concentrations of progesterone in the blood from the ovarian veins of a goat in late pregnancy, regardless of the presence or absence of a corpus luteum. However, they could not detect any progesterone in the uterine vein blood of the pregnant goat. It therefore seemed unlikely that the goat placenta was capable of producing any progesterone. Forbes (1955), using the Hooker-Forbes bioassay technique, detected "progestin" in the umbilical cord blood of goat foetuses during the last month of pregnancy. In three out of five goat foetuses examined, Forbes found the levels of "progestin" to be higher in the umbilical artery than in the umbilical vein, and he cites this as evidence that the foetus may produce progesterone which is subsequently removed by the placenta.

In the present study, it has only been possible to examine a very limited amount of goat material, but it is hoped to undertake a more extensive study in the near future. In an extract prepared from 1·2 kg. full-term goat placentas, no progesterone was detectable chromatographically. However, in 500 ml. arterial plasma obtained from a 16-week pregnant animal, 0·71 μg. progesterone/100 ml. plasma was detected. A 12 ml. plasma sample taken from the foetuses of the same animal failed to reveal any progesterone.

HAGOPIAN, M., PINCUS, G., CARLO, J., and ROMANOFF, E. B. (1956). *Endocrinology*, 58, 387.
HOOKER, C. W., and FORBES, T. R. (1947). *Endocrinology*, 41, 158.
HOFFMANN, F. (1954). *Zbl. Gynäk.*, 76, 2196.
HOFFMANN, F., and UHDE, G. (1956). III. Symposium German Soc. Endocrin., p. 212. Berlin: Springer
MAYER, G., and KLEIN, M. (1955). IIIe. Réunion des Endocrinologistes de langue française. Paris: Masson.
McDONALD, L. E., McNUTT, S H., and NICHOLS, R. E. (1953). *Amer. J. vet. Res.*, 14, 539.
MEITES, J., WEBSTER, H D., YOUNG, F. W., THORP, F. Jr , and HATCH, R. N. (1951) *J. Anim. Sci* , 10, 411.
NEHER, G M., and ZARROW, M. X. (1954). *J. Endocrin* , 11, 323.
PEARLMAN, W. H. (1957). *Biochem. J* , 65, 7P.
PHILIPP, E. (1936) *Zbl. Gynäk.*, 60, 86.
RAESIDE, J. I., and TURNER, C. W. (1955) *J. Dairy Sci.*, 38, 1334.
SHORT, R. V. (1956). *Nature, Lond.*, 178, 743.
SHORT, R. V. (1957). *J. Endocrin.* In press.
WIEST, W. G. (1956). *J. biol. Chem* , 221, 461.
ZANDER, J. (1955). *Klin. Wschr.*, 33, 697.
ZANDER, J (1956). III. Symposium German Soc Endocrin , p. 170. Berlin. Springer.
ZANDER, J., and SIMMER, H. (1954). *Klin. Wschr* , 32, 529.
ZANDER, J , and VON MUNSTERMANN, A M (1956) *Klin. Wschr.*, 34, 944.

DISCUSSION

Savard · I should like to compliment Mr Short on the very lovely study that he has done; I should also like to recount a few points of a rather

was administered intravenously over a 3-day period.

Summary and Conclusions

It is apparent that wide species differences exist in the hormonal mechanisms responsible for the maintenance of pregnancy. The high progesterone output of the placenta, as seen in the human, is not observed in any of the domestic animals considered here. In fact, the mare is the only other animal in which progesterone could be identified in placental tissue.

In the ewe, the maintenance of pregnancy after ovariectomy appears to depend on an extra-ovarian source of progesterone other than the placenta.

The biologically active progesterone metabolites found in the blood of some species probably make an important contribution to the overall progestational state of the animal. The continuing discrepancy between the biological determinations of blood "progestin" and the chemical determinations of blood progesterone indicates that other as yet unidentified progestins may occur in the blood.

Acknowledgements

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REFERENCES

Adler, A. A., De Fremery, P., and Tausk, M. (1934) *Nature, Lond.*, 133, 293.

Asdell, S A. (1946). Patterns of Mammalian Reproduction New York: Comstock.

Butt, W. R., Morris, P., Morris, C. J. O. R., and Williams, D. C. (1951). *Biochem J.*, 49, 434.

Denamur, R., and Martinet, J. (1955). *C.R. Soc Biol, Paris*, 149, 2105.

Duyvené de Wit, J. J. (1938). *Tijdschr. Diergeneesk.*, 65, 973.

[illegible] 309, 297.

[illegible] *Welt.*, 11, 745.

[illegible] *Progr. Hormone Res.*, 13. In press.

Grant, R. (1934). *Trans. roy. Soc. Edinb.*, 58, 1.

Short: I don't know anything about that. There is some slight evidence that progesterone may be bound. If you add it to plasma and do

to 60 per cent.
the chromatogr
plasma, and m
elution from th
that.

undoubtedly related to progesterone metabolism, particularly pregnanediol of the 20β type, and also an unidentified α : β-unsaturated ketone which Dr. Forbes later found to possess some progestational activity. Information on comparative aspects of progesterone production and metabolism is also available from urinary studies For

quite high in its radioactivity. Interestingly enough, the cholesterol

in the placenta. In our studies on the mare urine, we were able to iso-

fluid.

I suppose.

Borth: I can confirm that.

Bush: Mr. Short, is your extraction with ether done at room temperature?

Short: Yes, and I do it once with an equal volume and twice with half the volume of ether.

Bush: I presume that this addition of sodium hydroxide is preventing the usual very high lipid content that you would expect to get in such extracts.

CATECHOL HORMONES IN BLOOD

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The wide variations of the figures for adrenaline and noradrenaline concentrations in peripheral plasma indicate that the different methods employed do not give comparable results. While the measurement of the normal concentrations in the peripheral blood encounters great methodological difficulties, the conditions are more favourable for the estimation of catechol hormones in the suprarenal venous blood and in the blood of patients with catechol amine-producing tumours.

Suprarenal venous blood

Generally the catechol hormones occur in sufficiently high concentrations in the suprarenal venous blood to allow direct estimation by biological methods. During resting conditions the catechol amine level is low. It is, however, difficult to ascertain what should be regarded as the true basic level. In unanaesthetized animals several factors may enter which tend to increase the secretion from the gland. Even slight pain, immobilization, and other conditions, which induce mental or physical stress, are conducive to increased output of hormones from the gland. These factors seem to have been largely avoided by Satake, Sugawara, and Watanabe (1927) who collected blood samples from the suprarenal vein in the unanaesthetized, unrestricted dog after previous deafferentation of the operation field. They found a concentration of adrenaline equivalents of about 0·05–0·1 μg./ml. during rest. Part of this was presumably noradrenaline.

The surgical trauma in anaesthetized animals from which suprarenal venous blood is collected probably induces hypersecretion in most cases. Dunér (1953) found, in cats

same R_F value as progesterone in this system, namely, one that was reported recently from the Worcester Foundation by Dr. Pincus and co-workers, which they obtained from human placental perfusates. If in fact it is progesterone, then is there some mechanism which makes the adrenal take over progesterone production in the absence of the ovaries?

From 105 ml. adrenal vein plasma, taken at the 55th day of pregnancy, the concentration of this substance was about 5 µg. per cent plasma and at about 95 days of pregnancy it had gone up to about 7·5 µg. per cent. At this stage, the peripheral blood level of progesterone is about 0·3 µg. per cent, so although the adrenal vein concentration is 20 times that of the peripheral blood level, it is still obviously not enough to account for the entire amount of circulating hormone.

Parkes. Are you suggesting that the production of progesterone is peculiar to pregnancy?

Short: That we don't know. We have not had a chance to try the non-pregnant animal

Parkes When we obtained progesterone from adrenal extract waste products, as they were then, I should say that very few of these animals were pregnant.

Short: I think one has got to make a distinction between the content of progesterone within an adrenal, and the adrenal production of progesterone I don't think the two would necessarily go hand in glove

Savard. Mr. Short, have you any values on the daily urinary output of what might be considered as metabolites of progesterone in these other species? I have been particularly impressed with what we have encountered in the pregnant mare, where there seem to be great quantities of these substances in the urine, and with the very low levels circulating in the blood, one wonders just what the reason might be.

Short. In the mare I find about 250 µg progesterone per kg. placenta, and a mare placenta weighs several kg. As regards levels in the urine of other species, as Dr. Pearlman has mentioned, Dr Klyne has definitely established that there is no pregnanediol in cow urine. It has been detected in goat urine, but as far as I am aware, no systematic study has been done in pregnant and non-pregnant goats. Neither has anybody studied the pregnanediol levels in pregnant versus non-pregnant mares.

Savard: Dr. Klyne has failed to find the *pregn-3α:20α-diol* which would be the one encountered in human urine. I consider that within the error of our radioactive studies, this is quite absent from the pregnant mare. However, other allopregnanediols are present.

Short: I don't know which ones Dr. Klyne looked for in the cow, but I know that Dr. Turner has had some evidence to indicate that the cow probably excretes progesterone as an androgen in the faeces. If he gave increasing doses of progesterone he obtained an increase in the amount of androgen in the faeces, and I think Dr. Pearlman's finding of progesterone metabolites in the bile ties in with that story.

Peripheral blood

The constant finding of adrenaline and noradrenaline in urine indicates that the peripheral blood contains these hormones, even though the concentration is mostly so low as to present serious obstacles to their estimation. It can be assumed that the concentration level depends partly on the contribution from the suprarenals, which may be approximately calculated from the hormone level and the flow of the suprarenal venous blood. In the cat it seems permissible to use an approximate dilution factor of the order of 100; that is, a flow of 2 ml. blood from both suprarenals per minute corresponds to a total minute volume of 200 ml. When calculated on this basis and taking the "resting" hormone concentration as less than 0·1 μg. noradrenaline and 0·03 μg. adrenaline per ml. suprarenal plasma, the level in the peripheral arterial plasma would be less than 1 μg. noradrenaline and 0·3 μg. adrenaline per l. The figures are evidently only very approximate since they involve certain assumptions and may easily vary according to the secretory activity of the suprarenals. The possibility of a decrease in concentration during the passage through the lungs should also be considered. In this connection it should be emphasized that almost all of the adrenaline originates from the suprarenals, as concluded from observations of the adrenaline output in urine before and after adrenalectomy in man (Euler, Franksson and Hellström, 1954) and in the dog (Díaz, de la Barreda and Alcala, 1955).

A question which requires consideration is whether the constant inflow of adrenaline from the suprarenal blood causes a building-up of a higher adrenaline level than that corresponding to simple dilution. This is obviously the case if not all of the adrenaline is removed from the blood during one passage through the body, which can hardly be the case. On the other hand, there is no evidence that the resulting level is considerably higher than that calculated from the inflow. This conclusion is supported by the observation that adrenaline injected into the blood stream rapidly disappears.

subjected to light nembutal anaesthesia, about 0·10 µg. noradrenaline per ml. suprarenal plasma and about 0·03 µg./ml. adrenaline. Similar figures have been obtained by a number of workers (Table I).

Table I

ADRENALINE AND NORADRENALINE CONCENTRATION IN SUPRARENAL VENOUS PLASMA

Animal	*Adrenaline µg./ml.*	*Noradrenaline µg./ml*	*Author*
Cat (chloralose)	0 15	0 35	Holtz *et al.* (1952)
Cat (anaesth.)	0 095	0 061	Mirkin and Bonnycastle (1954)
Cat (chloralose)	0 047	0 18	Folkow and Euler (1954)
Dog (unanaesth.)	0 05–0 1	—	Satake (1954)
Dog (anaesth)	<0 01	<0 02	Lund (1951)
Rabbit (anaesth.)	0 10	0 021	Mirkin and Bonnycastle (1954)
Cat (nicotine)	4 8	5 6	Folkow and Euler (1954)

The concentration of catechol hormones in suprarenal blood can be raised by various means, such as direct or reflex splanchnic stimulation, and by various drugs with nicotine-like action, and by histamine. The catechol amine levels may then reach figures as high as 5 µg./ml. suprarenal plasma.

For the differential biological estimation of adrenaline and noradrenaline in suprarenal blood or plasma, direct assay on the cat's blood pressure and the chicken rectal caecum is satisfactory. The figures for adrenaline and noradrenaline can then be computed (Euler, 1949).

Summarizing, it can be stated that during conditions approaching rest as closely as possible the catechol hormone concentration in suprarenal venous blood is low, although it is nearly always measurable by direct bioassay.

during rest are available, some conditions are known in which the level is sufficiently increased so as to allow fairly accurate estimations.

In cases of phaeochromocytoma Lund has been able to measure the catechol hormone content of human venous plasma. The figures ranged between 10 and 100 μg./l. estimated by Lund's fluorimetric method (1952). Holzbauer and Vogt (1954) in a careful study found measurable quantities of adrenaline in venous human and canine blood after injections of insulin. The range found was 0·25 to 6·4 μg./l. in dog plasma, while the control level invariably was below 0·25 μg./l. in the dog and less than 0·06 μg./l. in man. The noradrenaline content of plasma was below 1 μg./l. which represents the threshold of the biological method used. Since the recovery of added amines was satisfactory it was felt that the figures observed are significant.

In a case of phaeochromocytoma studied by Euler, Gemzell, Strom and Westman (1955), blood samples were collected by catheterization of the vena cava and the plasma content estimated directly by biological methods. Figures ranging between 0·02 and 0·1 μg./ml. plasma were obtained, depending on the level at which the sample was drawn.

Assuming a similar relationship between the excretion of adrenaline in urine and the catechol hormone level in peripheral venous plasma as in cases of phaeochromocytoma (Euler *et al.*, 1953) and insulin hypoglycaemia (Euler and Luft, 1952), the resting level in plasma would be less than 1 μg./l.

Catechol hormones in effluent during stimulation of nerves to organs

Stimulation of the adrenergic nerves to an organ causes a release of the adrenergic nerve transmitter which is partly utilized and partly appears in the effluent blood. Direct estimations of the catechol amines in effluent blood have been made by Peart (1949), Outschoorn and Vogt (1952), Mann and West (1950), Mirkin and Bonnycastle (1954), Brown and Gillespie (1956) and others. In all cases noradrenaline was

The situation as regards the noradrenaline is more complex since it enters the blood stream both from the suprarenals and from the adrenergic nerve endings. Certain indirect information may again be derived from the observations on the catechol hormone excretion in urine in adrenalectomized patients. While the adrenaline excretion drops sharply after adrenalectomy, the noradrenaline is only little affected. Moreover, the noradrenaline excretion is normally higher than that of adrenaline both in the cat and dog and in man, although the suprarenal gland contains more adrenaline. It must be assumed then that most of the noradrenaline present in arterial blood is derived from the adrenergic nerve endings and carried by the venous blood. Some observations of Weil-Malherbe and Bone (1954) may be taken as support for this contention. Also for noradrenaline the rate of removal is probably such as to maintain the arterial blood level slightly higher than that corresponding to the inflow.

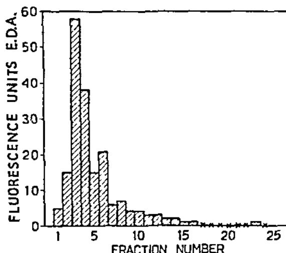
In the venous blood one may find widely varying noradrenaline figures depending on (1) the arterial blood level, (2) the rate of removal, chiefly in the capillary system, and (3) the release from adrenergic nerve fibres in the contributory area. Clearly the last-mentioned factor is of considerable importance for the significance of the values found by direct estimation. If venous blood is collected from an arm vein, the noradrenaline output may be very different from that obtained in blood from the liver, jugular or renal veins.

Noradrenaline figures in peripheral venous blood should for this reason be referred to the regions from which they are derived and should not be regarded as representative for the whole body.

In his study of the catechol hormones in peripheral human and bovine blood plasma Lund (1951) found that the level as a rule did not attain 1 µg./l.

Using column chromatographic separation of the catechol hormones of beef peripheral plasma Euler and Floding (1957) found values below 1–2 µg./l. While few reliable assays of the catechol hormone content of the peripheral venous blood

aline per l. plasma. A large number of papers have since been published in which this method or modifications thereof have been used. Similar figures to those found by Weil-Malherbe and Bone have been reported. In the search for the cause of the discrepancy between this method and the trihydroxyindole method it was noted that relatively large



amounts of 3 : 4-dihydroxyphenylacetic acid (dopac) normally occur in urine (Euler, Euler and Floding, 1955). Its appearance in urine suggested that it was also present in blood, and presumably in far higher concentrations than the catechol amines. Column chromatography of beef peripheral plasma after adsorption on aluminium oxide and elution showed the presence of dopac, while neither dopamine nor catechol amines could be demonstrated under the prevailing conditions (Figs. 1 and 2). Dopac is thus the quantitatively most important catechol compound in plasma, as it is in

found in concentrations of 0·01–0·1 μg./ml., while adrenaline, if present at all, occurred in smaller amounts. These results suggest that the contribution of adrenaline to the venous blood from sources outside the suprarenal medulla is small and inconstant, as is also borne out by the results from assays of the urinary output after adrenalectomy in man.

Comparison of assay methods

While direct biological estimation of the catechol hormone content in blood or plasma may give reproducible and reasonably accurate figures, this method is only applicable in exceptional cases and cannot be used as a standard method.

Biological estimation may be used after purification and paper chromatographic separation, a method, however, which is time-consuming and difficult. It has been used successfully by Holzbauer and Vogt (1954).

The chemical methods which have been most widely used are based on fluorimetric estimation of either the trihydroxyindole (THI) derivatives or the condensation products of catechol hormones with ethylene diamine (EDA).

The trihydroxyindole method was first applied to catechol hormones in plasma by Lund (1951) who obtained satisfactory results with plasma from cases of phaeochromocytoma, after injections of catechol hormones, and in peripheral blood from exsanguinated animals after adsorption of the catechol amines on aluminium oxide and subsequent elution. In normal beef or human plasma measurable quantities were only found occasionally, indicating that the normal content was below the threshold of the method, or about 1 μg./l. (adrenaline) and 2 μg./l. (noradrenaline).

By condensation of catechol and catechol derivatives with ethylene diamine, strongly fluorescent compounds are formed as shown by Natelson, Lugovoy and Pincus (1949). Weil-Malherbe and Bone (1952) applied this principle to plasma after purification on aluminium oxide. The figures found were rather higher than those obtained with the trihydroxyindole technique, about 1–2 μg. adrenaline and 4–5 μg. noradren-

Table II

3 : 4-DIHYDROXYPHENYLACETIC ACID (DOPAC) AND NORADRENALINE + ADRENALINE IN BEEF PLASMA (SERUM IN NO. 4) CALCULATED FROM FLUORESCENCE VALUES IN CORRESPONDING FRACTIONS OF COLUMN CHROMATOGRAM (ETHYLENE DIAMINE METHOD)

Sample No.	*Dopac* µg./l.	*Noradrenaline + adrenaline* µg./l.
1	15·5	<2
2	7 2	2
3	9 3	2
4	8 9	<1
6	19 6	<2
	Mean = 12 1	

The catechol figures in beef plasma, after adsorption on aluminium oxide and subsequent elution, obtained with the trihydroxyindole and the ethylene diamine methods are shown in Table III.

Table III

FLUORIMETRIC ESTIMATION OF CATECHOLS IN µG./L. BEEF PLASMA COLLECTED DURING EXSANGUINATION

Noradrenaline equivalents		*Dopac equivalents (ethylene diamine method)*
Trihydroxyindole method	*Ethylene diamine method*	
4 0	16	8·4
1 2	14	7 3
2 8	8 4	4 4
2 6	17 6	9 2
4 4	18 8	9·8
4 0	18 8	9 8
3 6	19 9	10·4
4 0	23	12
3 6	22	11 5
3 0	18 2	9 5
1 3	24	12 5
Mean = 3 1	Mean = 18·3	Mean = 9 5

urine. Since, on the other hand, dopac like other catechol substances gives a fluorescent condensation product with ethylene diamine, the ethylene diamine method cannot be regarded as specific for catechol hormones.

The amounts of dopac calculated from the fluorescence of the corresponding fractions in five plasma samples subjected to column chromatography are shown in Table II.

The condensation with ethylene diamine was carried out

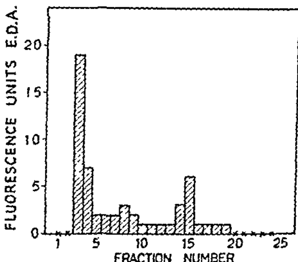


FIG. 2. As in Fig 1 Another beef plasma sample, to which dopamine had been added before adsorption.

with aliquots (0·05–0·3 ml.) of the column fractions containing 3 parts n-butanol saturated with 0·1N-HCl + 1 part 10N acetic acid. To the sample was added 0·7 ml. ethylene diamine + 0·5 ml. 2M ethylene diamine hydrochloride and water to 10 ml. After incubation for 20 minutes at 50° the fluorescence was read using a primary filter with maximum transmission at 436 mμ. and a secondary yellow-green filter with transmission limit about 500 mμ. The formation of fluorescent compounds with ethylene diamine has recently been studied by Burn and Field (1956).

adrenaline and take up added adrenaline in proportion to their ATP-content, but not noradrenaline. The amounts of adrenaline found in pig's platelets were $5 \cdot 8 \pm 0 \cdot 88$ mμg./10^8

Table IV

ADRENALINE AND NORADRENALINE CONCENTRATIONS IN HUMAN PERIPHERAL PLASMA OR SERUM ESTIMATED BY THE TRIHYDROXYINDOLE METHOD (THI) AND THE ETHYLENE DIAMINE METHOD (EDA)

Method	*Adrenaline* μg./l.	*Noradrenaline* μg /l	*Author*
Rat uterus	<0 06	<1 0	Holzbauer and Vogt (1954)
THI	<1	<1–1 5	Lund (1951)
THI	0 00	0 20	Valk and Price (1956)
EDA	0 083	1 3	Valk and Price (1956)
EDA	0 14	4 0	Manger *et al.* (1954)
EDA	0·4	2 1	Aronow *et al.* (1956)
EDA	1 2	5 3	Weil-Malherbe and Bone (1953)

platelets or, assuming a platelet count of 400,000 per $mm.^3$ (Hikmet, 1927) 4×10^{11} per l. The adrenaline amount may then be calculated as $23 \cdot 2$ μg./l. blood.

Summary

The fluorimetric method based on the formation of trihydroxyindoles may be used for the estimation of catechol hormones in blood plasma. The fluorescent compounds obtained by condensation of plasma catechols with ethylene diamine include dihydroxyphenylacetic acid which is present in high concentrations in plasma, rendering the method unspecific.

The normal adrenaline level of peripheral venous human plasma appears to be less than $0 \cdot 1$ μg./l. and that of noradrenaline less than $0 \cdot 5$ μg /l. During insulin hypoglycaemia the adrenaline level may be increased more than tenfold, and in patients with catechol amine-producing tumours the noradrenaline level may be increased more than 100-fold.

A fair agreement is noted between the dopac estimated after chromatographic separation and the dopac equivalents obtained by fluorimetric estimation using the ethylene diamine method. On the other hand, there is a large discrepancy between the noradrenaline equivalents calculated from the fluorescence observed with the two methods.

Valk and Price (1956) have recently drawn attention to some shortcomings of the ethylene diamine condensation method. As a result of their studies they conclude that the ethylene diamine method is not specific for noradrenaline and may not be so for adrenaline. They introduced two modifications of the Weil-Malherbe method prompted by the increased fluorescence of the adrenaline condensate in alumina eluate and the rapid decay of fluorescence in noradrenaline condensates when excited by light of 436 mμ.

It was further observed that omission of the fluoride-thiosulphate preservative did not alter the recovery of adrenaline, reduced that of noradrenaline by less than 20 per cent but diminished that of dopac by more than 50 per cent.

A comparison of the trihydroxyindole and ethylene diamine methods when applied to dog's arterial plasma showed good agreement between the adrenaline levels (about 1·8 μg./l.), while the noradrenaline concentration estimated by the trihydroxyindole method was only about 30 per cent of that obtained with the ethylene diamine method, (0·29 and 0·85 μg./l. respectively). The figures found by Lund (1951) in serum from exsanguinated dogs were 0·0–7·8 μg./l. adrenaline and 6·3–17·2 μg./l. noradrenaline.

In human venous plasma Valk and Price found 0·00 ± 0·005 (S.E.)μg. adrenaline per l. and 0·20 ± 0·097 μg. noradrenaline using the trihydroxyindole method. Table IV shows some figures of the catechol hormone concentration obtained with different techniques.

According to Weil-Malherbe and Bone (1954) catechol [illegible] Valk and Price [illegible] However, Born [illegible] atelets contain [illegible]

adrenaline and take up added adrenaline in proportion to their ATP-content, but not noradrenaline. The amounts of adrenaline found in pig's platelets were $5{\cdot}8 \pm 0{\cdot}88$ mμg./10^8

Table IV

ADRENALINE AND NORADRENALINE CONCENTRATIONS IN HUMAN PERIPHERAL PLASMA OR SERUM ESTIMATED BY THE TRIHYDROXYINDOLE METHOD (THI) AND THE ETHYLENE DIAMINE METHOD (EDA)

Method	*Adrenaline* μg./l.	*Noradrenaline* μg./l.	*Author*
Rat uterus	<0·06	<1·0	Holzbauer and Vogt (1954)
THI	*<1	<1–1·5	Lund (1951)
THI	0·00	0·20	Valk and Price (1956)
EDA	0·083	1·3	Valk and Price (1956)
EDA	0·14	4·0	Manger *et al.* (1954)
EDA	0·4	2·1	Aronow *et al* (1956)
EDA	1·2	5·3	Weil-Malherbe and Bone (1953)

platelets or, assuming a platelet count of 400,000 per mm.^{3} (Hikmet, 1927) 4×10^{11} per l. The adrenaline amount may then be calculated as $23{\cdot}2$ μg./l blood.

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The normal adrenaline level of peripheral venous human plasma appears to be less than $0{\cdot}1$ μg./l. and that of noradrenaline less than $0{\cdot}5$ μg./l. During insulin hypoglycaemia the adrenaline level may be increased more than tenfold, and in patients with catechol amine-producing tumours the noradrenaline level may be increased more than 100-fold.

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REFERENCES

Aronow, L., Howard, F. A, and Wolff, D (1956) *J. Pharmacol.*, **116**, 1.

Born, G. V. R, and Hornykiewicz, O. (1957). *J. Physiol.*, to be published.

Brown, G. L., and Gillespie, J S. (1956). *Nature, Lond.*, **178**, 980.

Burn, G. P., and Field, E. O. (1956). *Nature, Lond.*, **178**, 542

Díaz, C. J., Barreda, P. de la, and Alcala, R. (1955). *Rev clín. esp.*, **59**, 76

Dunér, H. (1953). *Acta physiol scand*, **28**, Suppl. 102.

Euler, Chr. v., Euler, U S v., and Floding, I. (1955) *Acta physiol. scand.*, **33**, Suppl 118, 32

Euler, U. S v (1949). *Acta physiol scand.*, **19**, 207.

Euler, U. S. v., and Floding, I. (1957). *Scand. J. clin. Lab. Invest*, **8**, 288.

Euler, U S. v., Franksson, C, and Hellstrom, J. (1954). *Acta physiol. scand.*, **31**, 6.

Euler, U S. v., Gemzell, C A, Strom, G., and Westman, A (1955). *Acta med. scand*, **153**, 127

Euler, U. S. v, and Luft, R. (1952) *Metabolism*, **1**, 528.

Euler, U. S. v., Lund, A, Olsson, A., and Sandblom, Ph. (1953). *Scand J. clin Lab Invest*, 5 122.

...tion Res., 2, 191.

...5, 222.

...umann. H.-J. (1952)

...harmacol., 9, 249.

Lund, ... Thesis, Copenhagen Munksgaard.

Lund A. (1952). *Scand. J. clin. Lab. Invest*, **4**, 263.

... Roth, ...341.

... 78, ...

Natelson, S., Lugovoy, J. L., and Pincus, J. B. (1949). *Arch. Biochem.*, **23**, 157.

Outschoorn, A. S., and Vogt, M. (1952). *Brit. J. Pharmacol.*, **7**, 319.

PEART, W. S. (1949) *J. Physiol.*, 108, 491.
SATAKE, Y. (1954) *Tohoku J. exp. Med.*, 60, Suppl (II).
SATAKE, Y., SUGAWARA, T., and WATANABE, M. (1927). *Tohoku J. exp. Med.*, 8, 501.
[illegible] *J. clin. Invest*, 35, 837.
[illegible] *Biochem. J.*, 51, 311.
[illegible] *Lancet*, 1, 974.
[illegible] *Biochem. J.*, 58, 132.

DISCUSSION

Vogt: Prof. von Euler, are the clearances of adrenaline and noradrenaline the same or not? Is, for instance, any evidence available from experiments in which a constant dose of adrenaline or noradrenaline was infused over a period of time and the amount excreted in the urine measured? The reason why I ask is that we believe that, at least under [illegible]

von Euler: I should say I almost feared that question, because I must admit that the clearance data are very scanty and I think I mentioned that they are only meant to give a general idea. However, I quite agree that it is necessary with the improved methods available really to work out in detail the clearance figures, and also to find out whether they apply at different concentration levels, which is also important

Harris: Prof von Euler, can you tell us what the present position is [illegible]

Vogt: I am afraid I have no precise information to give. Quite some time ago I tried to estimate adrenaline and noradrenaline in the blood of denervated animals and there was a very small secretion In adrenal vein blood of denervated animals the amounts were of the order of [illegible]

[illegible]

ment would have to be done after mid-brain section, but is there any

data that the catechol-amine content of the blood in, for example, the great vein of Galen or the straight sinus is increased after diffuse stimulation of the hypothalamus?

Vogt: From the point of view of the sympathin in the brain I have absolutely no information. The total quantity there, which is stored

the

rage

mu-

lation?

Vogt: You would exclude the adrenals by taking them out?

Harris: I was thinking of sectioning the midbrain and eliminating any descending influence on the adrenal medulla or sympathetic system.

Vogt: Well, if you found high figures that would mean that you would actually determine synthesis.

von Euler: We have an observation on a cat with one suprarenal denervated; for some reason the cat died during the night and in the morning we took out the suprarenals and analysed them. There was a normal content in the denervated one while the other was almost empty. So I think there must be a rather restricted secretion from the denervated adrenal in such conditions.

Singer: You mentioned that the secretion is low in Addison's disease.

the 3-methoxy derivative of
omocytoma.
would like to know what the
ditions. If I remember Born's
telets suspended in completely
f adrenaline were taking this

unphysiologically

amine. Now, in the experiments with ox serum to which you referred and in which you found these very low figures, you ought to have had everything present which platelets could have released; does the result not, at least for the ox, invalidate this view that the platelets contain a lot of noradrenaline?

von Euler: I have had exactly the same thought as Dr Vogt, and I think that when we were using serum in some of the experiments the adrenaline should have been released and then we should not have missed it. I also noticed that he used rather high concentrations of the amines when showing that the platelets are able to take up amines. But on the other hand, he demonstrated that pig platelets contain adrenaline without any treatment.

Vogt: But that was adrenaline and not noradrenaline.

von Euler: Only adrenaline.

Vogt. Yes, and that does not explain Weil-Malherbe's high results for noradrenaline.

Diczfalusy: Prof. von Euler, if I understood correctly, in adrenalectomized patients there is really no adrenaline demonstrated in the urine. Would it be possible to administer small amounts of adrenaline to such patients and estimate the amount excreted? This might then give us an answer to the metabolism of adrenaline. Have such experiments been carried out?

von Euler: They have not been carried out but I think it should be possible. It would certainly be interesting

GENERAL DISCUSSION

Morris: Dr. Parkes in his opening remarks suggested we went "*Back to Blood*". *I think it might be perhaps useful, at the end* of this week to look back to see if it is really worth while taking this as a main theme or not. I think it is, and for several reasons. The first Dr. Parkes himself gave in his opening address, that the hormone in blood in whatever state it is in, whatever concentration it is in, is in fact the active principle. I think Dr. Bush made the point

what is the minimum concentration of any hormone which is necessary to produce a biological effect, because the effect is usually limited by some rate process.

because I think that this indicates quite clearly that the assay itself is inadequate. As will be mentioned later, from the physical standpoint this is quite wrong; it will require quite a complex physical picture of the response to necessitate these transformations of the

ieme is that, in the case of the
he concentration of other hor-
tatic mechanisms. So it is quite
the case of the adrenal ACTH

circle, not only the ACTH concentration, but to understand the process thoroughly it is equally important to know the concentration of the cortical hormones. There are these feed-back effects, and

in order to assess the variables on one hormone very often it is valuable to know the concentration of another. I think this has a

iodine. That is only one example of a mechanism for which we cannot immediately see any rational cause. Another example was provided by the work Dr. Randle talked about, the inhibition of the response of the diaphragm to insulin by growth hormone and 17-hydroxycorticoids. Dr. Samuels has provided his evidence about the effect of the thyroid on corticoid production Dr. Szego gave a

trations we cannot use the kind of calculation Dr Pearlman talked about, a type of calculation which is a very important contribution to the study of the subject, because many of these numbers have at present only mathematical reality, and we cannot as yet resolve them into their components They are the first step in getting a sum pool or in effect what one might call a sum half-life, which later on with additional information could be parcelled into two or more different components. This again illustrates very nicely the importance of studies in blood

There are one or two things which are against work with blood. An important one was brought up by Prof. von Euler this morning. It is that the rate of release—and I think that one must use the word "release", we do not know really much about the synthesis of the hormone—from the cell in which it is forming may be quite likely to change from moment to moment. So an assessment made at one time is not necessarily valid a short time afterwards. This is the difficulty; when we measure, assuming we can measure, the same hormone in urine, we get what one might call an integrated result, integrated over a certain period of time, whereas the blood value is really a differential value In some ways the integrated value is also a useful type of information, especially in those cases where we may have rapid changes in rate of production and possibly in rate of utilization and destruction, and the blood level may be so variable as to be rather difficult to assess. I think that applies more particularly to certain hormones than others

Another point is the practical difficulty of measuring hormones in blood We have heard plenty to indicate how difficult this is; about the types of inhibitors which can occur, etc. Generally one has to deal with very much smaller quantities of material but that is

not an insuperable difficulty today. Nevertheless the practical difficulties are quite considerable compared to urine analyses, but I think that these difficulties will disappear with improved techniques and I do not think we need worry about that too much.

I have two more things to say. First, how are we going to investigate these problems? It is becoming quite a habit in colloquia to dismiss experimental methods and to say that the discussion should be confined to results This of course is nonsense. A conference which dealt entirely with results without any reference as to how they were obtained would be as unreal as the one which devoted

are in blood and how can we find out? It may perhaps be useful to trace an imaginary evolution of a method for the estimation of a hormone. What happens in the first place is that somebody finds some odd physiological result—it will often be quite bizarre—which at the time is quite inexplicable. Then the chemist comes along and says it would be quite nice to isolate the active principle. He always has contempt for the bioassay methods, but he learns after the first three years that the bioassay methods are much more vital than the techniques for isolation; the chemical isolation is easy relative to bioassay techniques. Then eventually a factor is isolated and is characterized and its constitution is established. People then begin to look for very specific properties in it and they

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In

the papers here we heard a very nice example of this trend of using a relatively non-specific method and using a greater purification of the fractions which are applied. I think this is a very important modern tendency. Instead of using a highly specific method on a very impure material, I think it is very much more rewarding to purify the material and use, in the first instance, a fairly specific method, either chemical or biological, and as one goes further on and wants to know more to use a less specific method. So finally one uses a quite non-specific method with characterization of the material, which you are looking for by other means, histochemical, chromatographical, and so forth If you do not do this, then you miss all the metabolites. I can give one simple example from an experience in my own laboratory. Dr. Farmer has just found, for instance, that corticotrophin in urine is partially in the oxidized biologically inactive form which you can reduce to restore its activity. About 50–75 per cent of the material is in this form. Now if you were

looking for only biological activity, as we were in the past, you miss this sort of thing. So I think that as research proceeds one should use specific methods of isolation, and less specific methods of final analysis. This way we will not miss the metabolites and we will not miss other forms that may be useful.

Dr. Roberts brought up the problem of how he needed to fractionate the test material further as the assay as he was using it was non-specific and he was trying out other fractions; it was mentioned that similar fractionation was necessary for the growth hormone assays. I think it is more and more clear that the way to tackle this type of problem is to apply good preliminary fractionation methods. If people should think that fractionation is difficult in such small quantities, I should advise them to look at Edstrom's beautiful work on the nanogram and picrogram determination of nucleic acids by electrophoresis It is a quite simple technique, and goes down to 10^{-8} µg. We haven't even started to use methods like that in endocrinology yet, so there is quite a lot of room for development still.

The other big problem which remains, and which we can only tackle *after the first is tackled, is in what form these substances are* present in blood. There are many ways of tackling it and Dr. Bush told us about one method, equilibrium dialysis, there must be many other possibilities

I certainly agree with Dr. Bush on his definition of the term "binding"; I think we should be very careful about using this word. "Binding" in the past generally meant something which

cules are of the order of about 1–2 kcal./mole at room temperature, and I should say the energy of complex formation should about double that to be considered a bond; this of course is an arbitrary definition, but it will include hydrogen bonding and distinguish from general forces of adsorption.

The future then lies very much in terms of these two lines of approach; on the one hand, investigation of the physicochemical state of these substances, and secondly the biological effects of the complexes. Finally, although we know a little about this for the steroids, we know nothing about it for the peptide and protein hormones, and I was very pleased to hear Prof. Heller tell us some first steps along this route. The steroids from this point of view of course are more easy to recognize and the techniques are easier, but both problems are equally important. I feel these are the most

important things, and alongside these, developments of analytical techniques will finally give us more knowledge of "Hormones in Blood".

Bush: Most of what I want to say is intended to stimulate dis- of

of

enough to find something like the dorsal muscle of the leech, or the guinea pig uterus, or the rat's colon available, so that we can often assay extremely small quantities of a hormone. However, when sensitivity is low, we have to consider whether the use of whole organs, and even worse, whole animals, is not somewhat wasteful. While such assays sufficed for much of the endocrinology that has gone on to-date, my impression is that the vast majority of bioassays we have heard of so far, with the possible exception of that for human chorionic gonadotrophin, are not sensitive enough to be worth considering seriously for blood assays. The sort of troubles that come up when the older methods of assay are used are that much of the material is adsorbed, or lost, in parts of the tissue which are not effectively responsive to the assay; in some methods the uptake of hormone

efficiency because of

of the experiment.

I don't think has been mentioned explicitly, is that some of these assays may lose efficiency by the presence of proteolytic enzymes

advance in methods may improve one's assay and how important specificity in this matter often is, I would cite the fact that Dr. Vogt and Miss Holzbauer found that even when adrenaline and noradrenaline had been separated on chromatograms one had to be careful to exclude the interference of histamine. It would seem utterly hopeless to attempt the biological assay of hormones without more attention to careful fractionation than seems usual. In

the field with which I am familiar, where the specificity and the reliability of the chemical assay of the substances concerned is far higher than one could hope for by the present bioassay methods, one nevertheless barks one's shins against problems of separation and fractionation every day.

Another example is the improvement in bioassays that can be gained by carrying out a simple adsorption step, such as the alumina adsorption of amines, the oxycellulose method for ACTH, and the adsorption methods for gonadotrophins. Another interesting example we heard of this morning was one from Mr. Short who found that by the apparently trivial addition of small amounts of sodium hydroxide to plasma, an extract which otherwise contains several grams of fat, making separation of the hormone extraordinarily difficult, is converted to an extract which is almost free from fat. I think it might be worth while looking into some of the

[illegible]

Bottari for TSH, may not be the assay methods of choice. Such [illegible] ...ity and may well

[illegible] ...vas made by Dr. [illegible] ...used that even in [illegible] ...eneral conclusions almost as much weight was given to results obtained in bioassays with apparently infinite limits of error as to those obtained in much more precise assays with a rather high index of precision. In discus- [illegible]

Prof. Gray mentioned this morning that there is a possibility of disease being caused by a change of peripheral consumption in the tissue. Another mechanism for endocrine disease could be a disturbed mechanism of regulation between the circulating target gland hormone and the trophic hormone. This problem can only be

studied in blood. I should like to hear whether other people also have information.

A patient in whom an observation was made was a man of 25 years who had a thyroidectomy for thyrotoxicosis, and who we saw in 1953 with local myxoedema. You have to realise that if a thyroidectomy is done for Graves's disease, you do not treat the

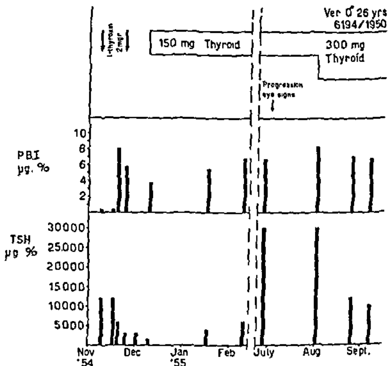


FIG. 1. (Querido).

disease, as the mechanism which caused the thyrotoxicosis is still there. In this case we were happy enough to find an excessive amount of thyrotrophic hormone in the blood when he was in a

4 and 6 µg. per ...

nicely. But I want you to note that it was still above the normal level. We later continued to give this patient thyroid powder and the PBI values remained practically normal between 4 and 8 µg. per cent.

We have repeated, at regular intervals, the estimations of thyrotrophic hormone in the blood, which suddenly after many months [illegible]

in the blood is concerned, does not function well.

These observations of course are rather difficult to make and I wonder whether other people have made similar observations in other endocrine disease, or have other data supporting the idea of a change in the feed-back mechanism.

Loraine: Prof. Querido, in the slide you showed of the patient with myxoedema the blood level of TSH was very high. Did you at the same time conduct any assays on urine? If so, do you have any data regarding the renal clearance of the hormone?

Querido: No, we haven't done any renal excretion. The only thing I can say is on the basis of animal experiments. When we were doing studies of the disappearance rate of TSH in rabbits, we were never able to find by methods employed in our laboratory any activity in urine, neither by adsorption or ultrafiltration procedures. But we have, of course, indications that it is fixed in the kidney and is cleared at a very fast rate. If heterologous thyrotrophic hormone is injected, you may find in 15 minutes 30 per cent of the material in the kidney with biological assay.

Referring to what Prof. Heller was saying about this kidney mechanism, we have tried to find out whether this mechanism could be of significance in patients. We did some rough experiments to try to block the kidney mechanism for extraction of thyrotrophic hormone. Injections with hydrolysed casein caused excessive swelling of the tubules cells, but still the kidney could fix the thyrotrophic hormone. We had to go as far as mercurial poisoning of the kidney to block the mechanism. So I think if one may transfer these observations on animals to humans, the reserve capacity of the kidney to pick up such minute quantities of biologically active material as thyrotrophic hormone must be in relation to other

would have to find a blocking substance. Taking the excretion of penicillin as an example: one can block its excretion quite effectively without damaging the kidney at all.

Querido. I agree.

Harris: Dr. Purves at a symposium here last year (1957. *Ciba Foundation Colloquium on Endocrinology*, **10**, 17) raised the point that Prof. Querido has just raised, that at least in some cases of hyperthyroidism there is an extra-thyroidal pathology. This is shown by normal or increased blood level of TSH simultaneously with an increased concentration of protein-bound iodine. In other words the feed-back mechanism has failed at some point which is outside the thyroid gland. There is also a physiological corollary to this which we (Harris, G. W., and Woods, J. W. (1957). *Ciba Found-*

[illegible]

Prof. Morris I think made the point of assaying both the target organ hormone and the pituitary trophic hormone in blood simultaneously. I think this is a very important point, certainly with regard to studies of the factors regulating pituitary activity.

Prunty: The scurvy guinea pigs are an example of the failure of this feed-back system. It was pointed out the other day that if you administered still more cortical hormones to them the feed-back mechanism seemed to be restored.

Sonenberg: As you may recall, Dr. Sayers considered that the concentration of hormone in blood, in his case ACTH, was a reflection of the amount excreted, presumably by the kidneys, the amount secreted by the pituitary, and the amount metabolized. I suggested to Dr. Farrell that there was another factor which others have raised here, namely, the volume of distribution. This is not particularly novel or original, but I was just reminded of some

[illegible]

formulated. So we and others have done experiments of labelling the blood or measuring the blood volume of various organs after

[illegible]

merely

volume of that organ is increased. Dr. Samuels has mentioned the

blood flow through an organ as being important, but this would be reflected in the metabolic function. What I am suggesting is that the levels of hormone in blood might be influenced merely by changing the partitioning of the blood in various tissues.

Heller: I should like to add another reason to those mentioned by Prof. Morris, [illegible] con-
nection with [illegible] hor-
mones in bloc [illegible] im-
portant in th [illegible] hor-
mones but they are so in many instances. I have always been surprised that some workers, mainly in clinical investigations, completely neglected the possibility of alterations in renal metabolic function. The metabolism of hormones is likely to be affected not only by renal disease involving cellular damage but also by haemodynamic changes originating in other parts of the body. Such changes may do what Dr. Sonenberg has just mentioned, namely alter the blood volume. But they are also bound to affect the metabolism of hormones in the kidney directly. It seems therefore [illegible]

being.

Harris: With regard to what Dr. Bush has said about assays, from time to time during this conference the question of assaying hormone in the venous blood flow of an organ has been raised; Dr. Samuels I think raised it with regard to the testis. It seems to me that, going back to Dr. Parkes' definition of a hormone as put forward at the beginning of the conference, the blood concentration of a hormone may tell you something about the organ that the hormone comes from, or something about the organ or tissues that [illegible]

moment is that of being able to obtain pituitary venous blood from [illegible]

manoeuvres for improving précision including the simple expedient of increasing the number of observations made, but I doubt whether it will be possible to improve the specificity of bioassay methods for protein hormones in blood. It would appear that time would be best spent in devising methods for the separation of protein hormones from blood rather than in improving the precision of biological ... hones will soon be obsolete.

vity of hormones and their
t the question of the inter-
le, the activity of growth
hormone in depressing the utilization of glucose by muscle is de- ... steroid whereas the activity
sion of glucose to fat
ty of adrenal steroid.
According to which ... ity we choose we may regard growth hormone as being relatively inert or highly active in the adrenalectomized animal.

Morris: I entirely agree on the use of these methods, as investigational tools—that is quite a different thing. If you can find a function which is non-linear that may give quite important information as to your system. I agree with Dr. Randle than an inaccurate assay may be very useful indeed, but I feel that if I had to deal with these conditions, I should take it as an indication one ... mental methods

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Samuels: I think interaction ... in explaining the high levels of the corticosteroids in pregnancy. We know that normally there seem to be adjustments in these levels; ... remain high as they do in pregnancy, and one wonders ... nature of the

the thyroxine-binding ...
in pregnancy, for instance, in regard to thyroxine. Some years ago ... with us, he had some data that the

the thyroid activity ...
in bioassay. What you are looking for in bioassay is the ...

measure sensitively and accurately a given substance. It may be that the natural situation is not the optimal for that particular process, and it might pay to find the optimal levels of these other functions.

I object a little to Prof. Morris's objection to "binding". When

Randle: I am anxious that Prof. Morris should not create a vacuum in the binding terminology. It is useful to have a term like "binding" which can be used loosely to describe an association between a hormone and other molecules even if we do not understand this association in a chemical sense. If we knew chemically what this association was then the term "binding" would no longer be necessary.

Morris: I think the difference between Dr. Samuels' and my views lies rather in the precision with which one system is defined and the other isn't. I was echoing Dr Bush's plea yesterday, and I do think that the term "binding" is used very loosely. If we have compound A and compound B and they form a complex, even in terms of an enzyme-substrate complex, then one should be able to measure the energi

more

term

Morris. I was thinking more specifically of the steroid field than of the protein field, but I think it still might apply. The term has been used far too loosely in the past.

Kellie. I am aware that when urine is used you get an average of the day's secretion; when blood is used you get an instantaneous [illegible] mpling. [illegible] er com- [illegible] blood, in many cases in microscopic quantities, and simultaneously you have relatively large amounts of inactive metabolites. There is some evidence that these metabolites are conjugated and are excreted. I should like to know what the feeling of the meeting is. What should we determine? Should we concentrate mainly on determining the minute amounts of the active hormone that are there and pay no attention to the conjugated metabolites which have little biological activity? I feel we cannot determine both, because as methods become increasingly complex, this becomes increasingly impossible.

Pitt-Rivers: I think it ought to be "on from blood"; the blood is actually only the route of the hormone from the thyroid to its target, and although it is a very important locus, it is not the objective of the hormone. The hormone is just going in the blood to somewhere, and this somewhere I think is one of the most important things to look into.

Savard: Speaking in the area of the corticosteroids and from a point of view of physiological significance, I just wonder if the blood determinations are giving us all that we had hoped to find. Admittedly the challenge to the analyst has been there, it has been met, and I think we will all agree that the methods are good. Mr. Short's contribution to-day on progesterone indicates that determinations of this hormone are reaching the same degree of reliability. However, I am a little disappointed in exactly what is in- [illegible]

volume may affect the rate of secretion. In one of the subjects

investigated we withdrew 600 ml. blood and the following day he showed a high aldosterone output. This may be a technical problem; we may just have to develop more sensitive methods and take less blood. In the case of cortisol we may have a more fundamental

On the subject of bioassay, it seems to me a pity that we have to discuss sensitivity and precision separately. I wonder if it

the lowest dose on an acceptable part of the dose response curve.

the extreme interest in the new clinical syndrome of primary aldosteronism. While ultimately a method for analysis of blood may be developed, the immediate goal should be the establishment of a good urinary method.

Samuels: I think Dr. Farrell's example of aldosterone is a good one to illustrate the limitations of observations in the urine. Dr. Tait's data would indicate that probably what you measure when you

active hormone. In most cases the free hormone is the physiologically active product in the blood We should also try to estimate the total hormone production by the body, which is a relatively simple procedure.

Morris I don't know how anyone knows what is the active form. You just cannot assume that; the free hormone may not be active hormone

Kellie I think I can exemplify that. You see, Dr. Pearlman, it could be that dehydro*epi*androsterone was secreted by the adrenal

gland, carried round the blood system as the sulphate and converted by the enzyme which Dr. Samuels has shown to be in the testis to testosterone *only* at the target site, and our attempts to measure testosterone may be quite misleading.

Morris: And the active substance might be a complex of testosterone with a protein.

von Euler: I haven't heard the word "utilization" yet, which is a little bit surprising, because whenever these things are discussed among endocrinologists, sooner or later, one comes to hear about "utilization" of the hormone, whatever it means. I suppose it just signifies the arteriovenous difference. But that again leads to the question whether one of the most important things here isn't actually to measure the arteriovenous difference, and of course the blood flow. I think that has not been sufficiently stressed. It could then be combined with the study of the products appearing in the organ itself. I think we would probably get a good picture of what happens in this way.

* * * * *

Parkes The idea of "back to blood" was not the result of any deep philosophical reflection on my part; it arose from long experience of

Zondek's demonstration that enormous amounts of oestrogen are found in the urine of the stallion This is now a very old and hackneyed

'e should
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e cellular

level.

and so on, but the fact remains that the whole problem of the estimation and characterization of the hormones in body fluids has now reached a formidable state of complication It is good to think that there is still scope for simplicity; Mr. Short, for instance, has given us an example of the way in which some happy stroke of inspiration may simplify methods and clarify results.

In the course of a conference on biological standards—held in 1938—Sir Henry Dale said that the ultimate aim of biological assay was self-extinction. His idea, of course, was that the information obtained by biological assay should sooner or later enable you to turn over to chemical methods. This undoubtedly happens, the chemical methods

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